THE ROLE OF 20-HYDROXYELOCOSATETRAENOIC ACID IN PREVENTING SALT INDUCED HYPERTENSION

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THE ROLE OF 20-HYDROXYEICOSATETRAENOIC ACID IN PREVENTING SALT INDUCED HYPERTENSION

A Thesis

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in the Department of Pharmacology

University of Saskatchewan

Saskatoon

By

Sowndramalingam Sankaralingam

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ABSTRACT

While there is a definite, positive correlation between salt intake and blood pressure, most animals and humans do not become hypertensive when exposed to high salt. Some however are "salt-sensitive". Sprague-Dawley (S-D) rats are generally considered to be salt-resistant, but when young may be more salt-sensitive. 20-hydroxyeicosatetraenoic acid (20-HETE), a ω-hydroxylated metabolite of arachidonic acid which is formed by the action of cytochrome 450 4A (CYP 4A) enzymes may be important in excreting a sodium load. Clofibrate, a lipid lowering agent and a peroxisome proliferator activated receptor α agonist induces CYP 4A and increases the renal synthesis of 20-HETE, and prevents hypertension in Dahl salt-sensitive rats. Based on the above, we hypothesized that when given a high salt intake, adult (52 weeks of age) S-D rats would increase renal 20-HETE synthesis, and excrete the sodium load. In contrast, the young (5 weeks of age) S-D rats would be unable to increase renal 20-HETE production, and therefore retain sodium and develop hypertension. Clofibrate should increase CYP 4A expression and 20-HETE synthesis in young animals. Groups of young rats were randomly assigned to receive either vehicle (20 mM Na₂CO₃) or 0.9% NaCl (saline) and half the number of animals in each group received clofibrate (80 mg/d) for three weeks. Adult rats were given either vehicle or saline to drink. Most parameters were measured after three weeks of treatment. Saline, but not vehicle induced a modest increase in blood pressure only in young rats (134 ± 2 vs. 115 ± 2 mmHg, p<0.001). Clofibrate did not affect blood pressure in the vehicle treated group. Interestingly, clofibrate not only prevented the increase in blood pressure in saline treated rats but demonstrated a hypotensive effect: blood pressure was lower than that seen in controls (102 ± 2 mmHg). A high salt intake increased CYP 4A mRNA expression in the renal cortex in the adult rats and increased 20-HETE
Younger animals showed decreased CYP 4A message and decreased urinary 20-HETE excretion. Clofibrate induced CYP 4A expression in the renal cortex of the saline treated young rats, and increased urinary 20-HETE levels. High salt intake also led to increased sodium retention, proteinuria and vascular superoxide anion production and decreased plasma total nitrite/nitrate levels in the young but not in the adult rats, all of which were prevented by clofibrate. Thus failure to increase renal synthesis of 20-HETE in the saline treated young rats led to sodium retention and hypertension which was prevented by clofibrate treatment. The adult rats increased renal 20-HETE synthesis on a high salt intake which aided in the excretion of sodium and prevented hypertension. We conclude that renal 20-HETE synthesis is important in excreting a sodium load. Failure to increase 20-HETE production may lead to salt-induced hypertension.
ACKNOWLEDGEMENTS

God blessed me with the opportunity to meet Dr. Thomas W. Wilson, my supervisor, who is well known not only for his wide scientific knowledge and in-depth clinical expertise but also for his pleasing personality and humane qualities, whom I would like to thank honestly and sincerely for having brought me to Canada and given me an opportunity to work on this project. It has been my good fortune!! I entered into the graduate program when I had only the vaguest idea of what research may be in hypertension. The confidence I have now in being able to critically analyze relevant scientific literature, design and carry out experiments, write scientific reports and present papers at conferences are solely due to the guidance and training of Dr. Wilson, to whom I am indebted in many ways. Dr. Wilson kindled in me an interest in research and honed my skills and guided me into the world of research in health sciences. I really adore his quick response and constructive criticism to the drafts of this thesis. His editing with appropriate language has contributed immensely to the final version of this thesis. Without his help this thesis might have been delayed inordinately. I thank him for sponsoring and supporting me to attend several scientific conferences which enhanced my confidence level in graduate studies immensely.

I thankfully acknowledge the colossal amount of assistance I received from my co-supervisor, Dr. Kaushik M. Desai who introduced me to the realm of endothelial function in hypertension. His extensive knowledge on nitric oxide and endothelial function, and enthusiasm as an educator introduced me to the length and breadth in this field. He has taught me techniques from as simple as operating a pipette to doing the complex in vivo experiments in anesthetized rats. I could walk into his office anytime to...
discuss the project and he has always had time to look into the details of my work and give invaluable timely advice. His patience and willingness to discuss even the minutiae of the different obstacles while working on this project were invaluable. His involvement in my graduate career is of great importance.

I would like to express my heart-felt thanks to the members of my thesis advisory committee, Dr. V. Gopalakrishnan (Chair), Dr. J.R. McNeill, and Dr. L. Wu for their valuable and timely guidance throughout my project. It has been a great privilege to have had Dr. V. Gopalakrishnan, a distinguished scientist, as chair of my thesis advisory committee whose guidance, support and critical inputs were important for this work in general. In particular, I appreciate the many impromptu EDHF & calcium lectures he conducted on any available writing surface, be it a scratch paper or a black board. I would like to take this opportunity to thank Dr. Wu for providing me with space to work in her laboratory and Dr. McNeill for allowing me to learn new techniques and perform some experiments in his lab.

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I would like to thank Mrs. Cindy Wruck for her morning greetings coated with advice and sense of humor about life, and Mrs. Irene MacDonald and Mrs. Donna Dodge for their friendship. My sincere thanks to Pinggang, Mahua, Xiaoxia and Urmila amidst whose friendship, support and good company this thesis gradually emerged. I am indebted to Dr. Xiaofan Qiu and Ms. Bobbie Duchek not only for their friendship but also for their technical expertise and guidance. I would like to thank Venkat and Pradeep, my friends from my boyhood days whose help and encouragement were instrumental for my coming to Saskatoon.

I would like to thank my parents, Mrs. S. Saraswathi and Mr. S. Sankaralingam for encouraging me to undertake my studies at Saskatoon and whose blessings are invaluable for my graduate studies; as also my sister S. Renuka for her love and affection.

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The kind and unstinted support of Dr. Sandy Davidge, my supervisor at the University of Alberta and of all my colleagues at Edmonton, which enabled me to complete this thesis, is thankfully acknowledged. I wish I could mention each individually, but will organize a large party instead in thankfulness.
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<tr>
<td>[Ca$^{2+}$]$_i$</td>
<td>Intracellular calcium</td>
</tr>
<tr>
<td>1-ABT</td>
<td>1-Aminobenzotriazole</td>
</tr>
<tr>
<td>17-ODYA</td>
<td>17-Octadecynoic acid</td>
</tr>
<tr>
<td>20-HETE</td>
<td>20-hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ADMA</td>
<td>Asymmetric dimethyl arginine</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BP</td>
<td>Blood Pressure</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic 3', 5'-adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic 3', 5'-guanosine monophosphate</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P-450 monooxygenase</td>
</tr>
<tr>
<td>Dahl SS</td>
<td>Dahl salt-sensitive rats</td>
</tr>
<tr>
<td>Dahl SR</td>
<td>Dahl salt-resistant rats</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy ribonucleic acid</td>
</tr>
<tr>
<td>DOCA</td>
<td>Deoxycorticosterone acetate</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium derived relaxing factor</td>
</tr>
<tr>
<td>EET</td>
<td>Epoxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ET</td>
<td>Endothelin</td>
</tr>
<tr>
<td>FW</td>
<td>Forward</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium</td>
</tr>
<tr>
<td>K&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>Calcium activated potassium channel</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Potassium phosphate</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulphate</td>
</tr>
<tr>
<td>Mmol</td>
<td>Millimole</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>Sodium carbonate</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Sodium phosphate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate dehydrogenase</td>
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<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>NO&lt;sub&gt;x&lt;/sub&gt;</td>
<td>total nitrite/nitrate</td>
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\begin{itemize}
\item $O_2$ \hspace{1cm} Oxygen
\item $O_2^-$ \hspace{1cm} Superoxide
\item ONOO$^-$ \hspace{1cm} Peroxynitrite anion
\item NOS \hspace{1cm} Nitric oxide synthase
\item PPAR \hspace{1cm} Peroxisome proliferator activated receptor
\item PT \hspace{1cm} Proximal tubule
\item RNase \hspace{1cm} Ribonuclease
\item ROS \hspace{1cm} Reactive oxygen species
\item RV \hspace{1cm} Reverse
\item S-D \hspace{1cm} Sprague-Dawley
\item SEM \hspace{1cm} Standard Error of Mean
\item SHR \hspace{1cm} Spontaneously hypertensive rat
\item SHR-SP \hspace{1cm} Spontaneously hypertensive rat-stroke prone
\item SOD \hspace{1cm} Superoxide dismutase
\item TALH \hspace{1cm} Thick ascending loop of Henle
\item THF \hspace{1cm} Tetrahydro furan
\item VSMC \hspace{1cm} Vascular smooth muscle cells
\end{itemize}
**1.0 INTRODUCTION**

Hypertension can be defined as the level of blood pressure at which the benefits of treatment outweigh its costs and hazards. The usual operational definition is a sustained blood pressure of over 140/90 mm Hg (Chobanian et al., 2003). The World Health Report 2002 has identified high blood pressure as one of the ten leading risk factors globally and regionally in terms of burden of disease. High blood pressure results in 64 million Disability-Adjusted-Life Years.

**1.1. Salt –sensitivity of blood pressure**

Salt sensitivity, an important factor associated with hypertension and its complications has both environmental and genetic components. It appears to be more widely prevalent among African Americans than other ethnic groups in America (Peters & Flack, 2000).

World epidemiologic studies have demonstrated that habitual ingestion of high levels of dietary salt (NaCl) is associated with increased blood pressure (Cooper et al., 1997; Stamler, 1997). Conversely, societies with habitually low salt intake have little or no hypertension and show little rise in blood pressure with age.

**1.1.1. Definition**

Peters and Flack offered a definition: “Conceptually, salt sensitivity is defined as an increase in blood pressure in response to increased sodium ingestion and/or a decrease in blood pressure when sodium intake is reduced, with the degree of change exceeding that attributed to directionally appropriate random blood pressure variation”. Salt sensitivity
occurs as a result of alterations in kidney function that require higher arterial pressure to maintain "steady-state" homeostasis, which is reflected in a "resetting" or a shifting of the pressure-natriuresis curve to the right. (Luft et al., 1997; Cowley et al., 1997; Sullivan et al., 1991).

Operational definitions of salt sensitivity have been arbitrary and varied. Some researchers define salt sensitivity as a fixed, arbitrary change in pressure (i.e., more than a 3 mm Hg directionally appropriate change), while others define it as a directionally appropriate proportional change (i.e., a 10% change in pressure) (Sullivan et al., 1991).

1.1.2. Factors associated with salt-sensitivity of blood pressure in humans

There are five broad categories of factors consistently associated with salt sensitivity.

1.1.2.1. Demographic factors

In humans, there is a direct, positive relationship between age and salt sensitivity, with increasing salt sensitivity observed in the elderly (Rocchini et al., 1989, 1992, 1995). Weight may also be responsible for the gender differences noted in blood pressure response to sodium. As a result, women are usually found to be more salt-sensitive than men.
1.1.2.2. Race vs. Social factors

The incidence of salt sensitivity of blood pressure appears to be more prevalent in young, old, normotensive, and hypertensive African Americans than Caucasians (Luft et al., 1979, 1991; Falkner et al., 1986; Weinberger et al., 1986). This increased prevalence of salt sensitivity in African Americans has been demonstrated in studies using intravenous NaCl loading as well as in studies using diet that differ in sodium levels (Luft et al., 1991, 1997; Sullivan, 1991). Salt sensitivity is so prevalent among the African Americans, that it is considered to be a "hallmark" of black hypertension, (Svetkey, 1997).

1.1.2.3. Renal Function Factors

Individuals with renal insufficiency or renal damage are more likely to be salt-sensitive, and salt-sensitive subjects are more likely than salt-resistant subjects to have kidney damage. This, in turn, is characterized by an attenuated increase in glomerular filtration rate in response to salt loading, and microalbuminuria (Weinberger, 1996; Luft et al., 1991, 1997; Sullivan, 1991). In addition, salt-sensitive individuals, particularly women, have suppressed circulating renin levels (Stein & Black, 1993; Weinberger, 1996). These alterations in renal function result in a shift of the pressure-natriuresis curve to the right (see below), indicating that salt-sensitive persons require higher systemic pressure to maintain steady-state sodium homeostasis (Flack et al., 1991; Weinberger, 1996; Sullivan, 1991).
1.1.2.4. Hormonal factors

Salt-sensitive subjects also demonstrate differences in hormone levels that are involved in sodium homeostasis. When compared to the salt-resistant subjects, the salt-sensitive subjects have higher levels of norepinephrine, a hormone that is implicated in sodium retention and lower levels of dopamine, a hormone that promotes sodium excretion. Both plasma norepinephrine concentrations and urinary sodium excretion are higher among salt-sensitive than salt-resistant subjects (Weinberger, 1996; Luft et al., 1997; Gill et al., 1991). Similarly, a deficiency of kallikrein, a renal natriuretic hormone has been reported in salt-sensitive individuals.

1.1.2.5. Dietary habit factors

Lower potassium and calcium intake was also associated with sodium retention, and increased blood pressure (Luft et al., 1997; Weinberger et al., 1993). In summary, hypertension requires an adequate (high) salt intake in a susceptible individual.

1.2. ROLE OF KIDNEY IN HYPERTENSION

There is convincing evidence to suggest that renal function is altered in hypertension. However, whether the observed changes in renal function are a consequence or the primary basis of hypertension remains unresolved. It has been established beyond doubt, that the kidney is progressively damaged as a long-term consequence of hypertension. The following lines of evidence indicate that there is alteration of renal function before the development of hypertension (Cowley AW Jr & Roman, 1996).
Several studies have confirmed the dominant role of kidney in the long term control of blood pressure. In almost every experimental model of hypertension, some abnormalities in renal excretory function precede the onset of hypertension, and global renal damage. A higher level of arterial pressure is required by the kidney to excrete sodium and water in animal models of hypertension. Further, antihypertensive drugs promote sodium and water excretion and decrease the slope of the pressure-natriuresis curve. During impairment of renal excretory function, an increase in arterial pressure is essential to restore the fluid and electrolyte balance. Blood pressure follows the kidney in renal transplantation studies in humans and genetic models of hypertension in rats (Cowley AW Jr & Roman RJ, 1996).

1.3. Pressure-natriuresis

An elevation of renal arterial perfusion pressure results in increased excretion of sodium and water. In normal animals, an elevation in arterial pressure would increase sodium and water excretion by pressure natriuresis. Because sodium and water intake remains relatively fixed, the increase in sodium and water excretion gradually lowers blood volume sufficient enough to restore arterial pressure to control levels. The pressure natriuresis relationship exhibits either a slope reduction or is shifted toward a higher set point, in experimental models of hypertension. A lower slope is indicative of salt-sensitivity. When blood pressure is reduced for any reason, sodium and water are retained until the pressure returns to control and restores sodium and water balance.
Figure 1. Schematic representation of the pressure-natriuresis relationship. The horizontal line indicates the usual sodium intake. In normal animals, an increase in arterial pressure will increase sodium and water excretion via pressure natriuresis. In hypertensive states, the pressure-natriuresis relationship exhibits a decrease in slope or is shifted in a parallel manner toward higher pressures.

1.3.1. Pressure–natriuresis relationship in Dahl SS rats

The Dahl salt-sensitive rat (Dahl SS) was developed from outbred Sprague-Dawley rats and later refined to an inbred strain with both S and salt-resistant (R) types (Rapp, 1993). The slope of the pressure-natriuresis relationships in Dahl S rats is approximately half of that measured in Dahl salt-resistant (Dahl R) rats. The abnormal pressure-natriuresis relationship in the Dahl S rats is due to enhanced reabsorption of sodium and chloride in the thick ascending loop of Henle (Roman et al 1991a; Kirchner et al., 1985, 1986 &
1990). After Dahl S rats are fed a high-salt diet for 2 weeks, they develop hypertension that cannot be reversed by returning them to a low-salt diet. The development of sustained hypertension in these animals is accompanied by renal damage and decreased GFR that further shifts the pressure natriuresis curve to the right, which explains the persistence of hypertension in Dahl S rats even after they are returned to a low salt diet.

1.4. Salt-sensitivity in Sprague-Dawley rats

The seminal works of Lewis K. Dahl provided evidence for the fact that sodium chloride plays a major role in the regulation of BP. Some S-D rats show a dose dependent increase in SBP when given a high salt diet. Moreover, the earlier the introduction of a high salt diet, the sooner and higher was the increase in BP. However, he observed that not all the S-D rats developed an increase in BP and in those that developed an increase in BP there were wide variations. Those rats that developed an increase in blood pressure with a high salt diet were called ‘salt-sensitive” while those that did not were known as ‘salt-resistant”. The inbreeding of the salt-sensitive rats led to the development of the Dahl SS/Jr rats while the other group led to the Dahl SR/Jr rats (Rapp 1993). These observations were confirmed by Zicha et al who showed that the young rats are more salt sensitive than the adult ones. The natriuretic capacity of the S-D rats increases with age, while the salt-sensitivity of blood pressure decreases with age. These were attributed to an “immature atrial natriuretic peptide system”. However, the precise mechanisms for the young S-D rats being more salt-sensitive are uncertain.
1.5. Arachidonic acid (AA) metabolites in modulating vascular tone

Arachidonic acid (AA) is a 20-carbon long chain polyunsaturated fatty acid. It has been well established that cyclooxygenase (COX) and lipooxygenase (LOX) enzymes metabolize arachidonic acid (AA) to prostaglandins, prostacyclin, thromboxane, and leukotrienes that play a role in the modulation of renal and pulmonary function, vascular tone, and inflammatory response. In the 1980’s a third pathway for the metabolism of AA was identified. Capdevila and others reported that AA is also metabolized via cytochrome P-450 (CYP) enzymes in the liver and kidney to EETs and DiHETEs. Subsequent work demonstrated that 19- and 20-HETEs are formed in some extrahepatic tissues by the action of CYP enzymes, particularly CYP A2. Iwai and Inagami (1991) reported that the “S3 gene”, subsequently found to be identical to the CYP 4A2 gene was overexpressed in the kidney of young spontaneously hypertensive rats. Subsequently, Sacerdoti et al (1988) and Omata (1992) reported an increased production of 20-HETE in the kidneys of the SHR. These observations suggested that an elevation in the renal production of 20-HETE may play a role in the development of hypertension. Alternatively, CYP4A mediated 20-HETE production could be a compensatory mechanism in hypertension.

1.5.1. Metabolism of AA by CYP 450 enzymes

CYP genes encode cytochrome P 450 enzymes. These CYP enzymes catalyze NADPH-dependent oxidation of drugs and endogenous ligands such as steroids and fatty acids (Nelson et al., 1996; Scarborough et al., 1999). NADPH reductase and CYPb5 (NADH/NADPH oxidase) serve as co-factors for the cytochrome P 450 group of enzymes (Capdevila et al., 1981). The CYP 4A and CYP 2C groups produce the most
important compounds with respect to hypertension. Hence they will be discussed in detail.

Arachidonic Acid

COX

PGI₂
PGE₂
PGF₂α
PGD₂

CYP 450

EETs

20-HETE

LOX

Leukotrienes

Figure 2. Pathways for the metabolism of arachidonic acid.

Arachidonic acid is metabolized via the cyclooxygenase to yield prostaglandins, prostacyclin and thromboxane; lipooxygenase to produce leukotrienes; and cytochrome P-450 (CYP) enzymes to form 20-HETE and EETs.

1.5.1.1. ω-Hydroxylases

Several enzymes of the CYP 4A, 4B and 4F families have been reported to catalyze the ω-hydroxylation of fatty acids. Some of the isoforms in these families generate 20-HETE when incubated with arachidonic acid. Human liver and kidney express CYP 4A11, 4F2, 4F11, and 4F12. In the human kidney, CYP4F2 appears to be dominant isoform in the formation of 20-HETE (Powell et al., 1998). The observation that antibody to CYP4F2
reduced 20-HETE formation by microsomes prepared from human kidney by 67%, while antibody to CYP4A11 reduced the formation of 20-HETE by only 32% suggests that CYP 4F2 is the predominant isoform in forming 20-HETE in human kidney. Atleast four isoforms of the CYP4A gene family, namely CYP4A1, 4A2, 4A3 and 4A8, have been shown to be expressed in the liver, kidney, and brain of rats. When incubated with arachidonic acid, recombinant CYP4A1, 4A2, and 4A3 enzymes but not 4A8 produce 20-HETE (Nguyen et al., 1998; Stromstedt et al., 1990 & 1994; Wang et al., 1996). However, Hoch et al (2000) have shown that CYP4A8 does form 20-HETE in small quantities. The CYP4A1 enzyme catalyzes AA 10 times faster than CYP4A2 or 4A3. CYP4A2 is the isoform that is constitutively expressed in the liver, kidney and vasculature of rats. The protein levels and mRNA expression of CYP 4A1 are relatively low in these tissues and can be induced by fibrates such as clofibrate (Ito et al., 1998, 1999; Gebremedhin et al., 2000). The mRNA expression of CYP4A2 and 4A3 isoforms have been reported in small arterioles in the kidney of rats (Imig et al., 1996; Ito et al., 1999)

1.5.1.2. Epoxigenases

Epoxigenases catalyze the formation of EETs from AA. CYP enzymes of the P450 1A, 2B, 2C, 2D, 2E, 2J, and 4A families have all been reported to catalyze the formation of EETs in various tissues and species. Studies using sulphaphenazole, a CYP2C9 inhibitor suggests that CYP 2C9 accounts for about 50% of the epoxigenase activity in human liver. Human endothelial cells express enzymes of the CYP2C8, CYP2C9 that produce EETs (Fisslthaler et al., 1999). EETs dilate human coronary arteries (Miura et al., 1998,
They act by hyperpolarizing VSM by opening K\(^+\) channels and could be considered to be the endothelial-dependent hyperpolarizing factor (EDHF). CYP2C11 and 2C23 are expressed in the kidney of rats. CYP2C23 is the predominant epoxygenase isoform that forms EETs in the kidney of rats (Holla et al., 1999).

1.5.1.3. Metabolic Fate of EETs and 20-HETE

Soluble epoxide hydrolases catalyse the conversion of EETs to their corresponding DiHETEs thus limiting the actions of EETs. EETs and 20-HETE undergo β-oxidation to form 18- and 16- carbon compounds that are biologically less active. EETs and 20-HETE are also metabolized by cyclooxygenase (COX) to vasoconstrictor endoperoxides or to vasodilator prostaglandins and prostacyclin analogues. EETs and 20-HETE are reincorporated into membrane phospholipids pools and can be released again by agents that activate phospholipases. These fatty acids are also bound to plasma proteins and hence their biological activity is limited in circulation.

![Figure 3. Metabolic fate of 20-HETE](image-url)
20-hydroxyeicosatetraenoic acid (HETE) is metabolized by β- Oxidation to 18- and 16-carbon derivatives, that are less biologically active. 20-HETE is also metabolized by cyclooxygenase (COX) to vasoconstrictor or to vasodilator prostaglandins or is reincorporated into membrane phospholipid pools.

1.5.2. Regulation of CYP Enzymes:

1.5.2.1. CYP Inhibitors:

The CYP inhibitors can be classified as selective and non-selective inhibitors. The recent availability of relatively selective inhibitors of CYP have advanced the understanding of the actions of the CYP metabolites.

1.5.2.1.1. Non-selective Inhibitors

The release of AA from membrane phospholipids can be blocked by nonspecific phospholipase inhibitors such as quinacrine or heparin or by the more specific inhibitor of phospholipase A2, arachidonyl-trifluoromethyl ketone (AACOF₃). β-diethyl-aminoethylidiphenylpropylacetate (SKF-525) binds to heme in CYP enzymes (Capdevilla et al., 1992) and reduces CYP activity by diminishing the availability of heme for incorporation into CYP enzymes thus reducing the formation of EETs and 20-HETE. However, these agents are non-specific and reduce the activity of all CYP enzymes, including other heme-containing enzymes such as nitric oxide synthase (NOS). Suicide-substrate CYP inhibitors like 1-aminobenzotriazole (ABT) bind to, and inactivate CYP isoforms that utilize these compounds as substrates and appear to be more specific in inhibiting CYP. 1- ABT (50 mg/kg ip) selectively inhibited enzymes of the CYP4A
family and reduced the formation of 20-HETE in the kidney of rats (Su et al., 1998). However, others have reported that 1-ABT also blocks the activity of other CYP isoforms (CYP2B, 1A, 2C) that produce EETs in the liver and lung (Knickle et al., 1992; Mathews et al., 1985). ABT (50 mg/kg ip) completely blocks the formation of EETs, DiHETEs, and 20-HETE in the kidney of rats within 2 h and reduces the 24-h excretion of 20-HETE by about 50% (Maier et al., 2000). It also reduces the formation of 20-HETE and EETs in the liver and lung by ~50%. Even though ABT is nonselective in blocking the formation of EETs, its orally active property and being well tolerated by rats for more than 2 weeks (Meschter et al., 1994) makes it a suitable choice for in vivo studies. Above all, it is readily available (Sigma) and inexpensive. Another relatively specific agent is 17-octadecynoic acid (17-ODYA) thought to be a specific suicide substrate inhibitor of the CYP4A enzymes that metabolize AA to 20-HETE (Muerhoff et al., 1987; Ortiz et al., 1984). 17-ODYA irreversibly blocks the formation of 20-HETE at concentrations of 1 μM (Zou et al., 1994). Unfortunately, it also blocks EET formation in other tissues (Zou et al., 1994). Finally, it is almost insoluble in water and very expensive, making in vivo studies difficult and expensive.
Cell membrane Phospholipids

\[ \text{PLA}_2 \]

Arachidonic Acid

\[ \omega\text{-hydroxylase} \quad \text{Epoxynase} \]

\[ \text{ Clofibrate } + \quad \beta\text{-napthaflavone}\]

\[ \text{ Fenofibrate } = \text{ Phenobarbitol} \]

DDMS

DDBB

HET 0016

\[ \text{ ABT } \quad \text{17-ODYA} \]

\[ \text{ 20-HETE} \]

\[ \text{VASOCONSTRICTION} \]

\[ \text{EETs} \]

\[ \text{VASODILATION} \]

Figure 4. Inducers and inhibitors of arachidonic acid metabolism

Dashed arrows represent inhibition; Plus sign represents induction

1.5.2.1.2. Inhibitors of the synthesis and actions of 20-HETE

Dr. J.R. Falck synthesized 12,12-dibromododec-11-enamide (DBDD) and DDMS. These compounds selectively block the formation of 20-HETE (Alonso-Galicia et al., 1997; Wang et al., 1998). At concentrations less than 10 \( \mu \text{M} \), these compounds competitively inhibit the formation of 20-HETE by renal microsomes, while reducing epoxynase.
activity by only 10-20% (Alonso-Galicia et al., 1997). This relatively select effect is lost at higher concentrations. The fact that these inhibitors are fatty acids that avidly bind to plasma proteins, limits their usefulness in in vivo studies. HET-0016 (N-hydroxy-N-(4-butyl-2 methylphenyl)formamidine) appears to be the most specific inhibitor of 20-HETE formation currently available (Kehl et al., 2001; Miyata et al., 2001). This compound selectively inhibits the formation of 20-HETE at a concentration <10 nM. Recently, the use of antisense cDNA oligonucleotides directed against CYP4A isoforms serve to specifically target each of the CYP 4A isoforms. This could lead to the determination of the contribution of each of these isoforms in the regulation of renal function and vascular tone. Using anti-sense oligonucleotides, it might be possible to chronically block CYP isoforms. Whether other CYP 4A isoforms would compensate for reduced activity of the isoform being blocked is not known.

1.5.2.2. CYP Inducers
The expression of CYP enzymes can be induced by both endogenous substances and exogenous agents. Fatty acids, and steroids including glucocorticoids, mineralocorticoids and progesterone, increase the renal and pulmonary CYP 4A protein expression (Lapuerta et al., 1998; Masters et al., 1991; Simpson, 1997). Hypolipidemic agents such as clofibrate induce the hepatic and renal expressions of CYP 4A1 and 4A3 and possibly CYP 4A2 (Aoyoma et al., 1990; Gibson, 1989; Hardwick et al., 1987; Roman et al., 1993; Simpson, 1997) by inducing peroxisome-proliferator activated alpha (PPAR-α) receptor (Green, 1995; Kroetz et al., 1998). Chronic exposure to NO increases the expression of CYP4A mRNA and protein in the liver and kidney (Ito et al., 1999; Sewer
et al., 1996 & 1997). Interestingly NO itself inhibits the formation of EETs and 20-HETE (Alonso-Galicia et al., 1997 & 1998; Oyekan et al., 1999; Sun et al., 1998). In contrast, NO downregulates the formation of CYP2C isoforms (Lopez-Garcia et al., 1998; Sewer et al., 1996 & 1997). Almost all vasoconstrictor agents such as Ang II activate phospholipases and increase the synthesis and release of 20-HETE in the kidney and VSM cells (Carroll et al., 1996 & 1997; Croft et al., 2000; McGiff et al., 1999; Oyekan et al., 1997 & 1998; Vazquez et al., 1995). Renal 20-HETE formation is also altered by dietary sodium (Holla et al., 1999; Ito et al., 1999; Makita et al., 1994 & 1996; Oyekan et al., 1999; Hoagland et al., 2003) and potassium intake (Carroll et al., 1990; Wang et al., 2001; Zhang et al., 2000).

1.5.3. Actions of 20-HETE

20-HETE exerts its actions in both the vascular smooth muscle cells where it modifies vascular tone and in renal tubular cells where it prevents reabsorption of sodium. Apart from these, 20-HETE also functions as an oxygen sensor, mediates myogenic responses and mediates the vasoconstrictor actions of other vasoactive molecules such as ET-1. The vascular smooth muscle and the renal actions of 20-HETE are described in detail.

1.5.3.1. Vascular actions of 20-HETE

20-HETE evokes vasoconstriction via inhibition of the large conductance $\text{K}_{\text{Ca}}$ channel. In VSMC, inhibition of 20-HETE with 17-ODYA activated the large-conductance $\text{K}_{\text{Ca}}$ channel an effect that was completely abolished by exogenous addition of 20-HETE (Harder et al., 1994). 20-HETE constricts mesenteric, renal and skeletal muscle arterioles
(Chu et al., 2000; Roman et al., 1993; Frisbee et al., 2001). After inhibition of the production of endogenous 20-HETE by 17-ODYA, exogenous 20-HETE reduced the diameter of the resistance vessels. The vasoconstrictor response to 20-HETE in peripheral arteries of rats and rabbits is partly blocked by indomethacin or an endoperoxide receptor antagonist, suggesting a role for COX metabolites of 20-HETE acting through the TP receptor, in its biological function (Escalante et al., 1993). 19-HETE has been shown to be a competitive antagonist of 20-HETE, suggesting the presence of receptor for 20-HETE in VSMC (Alonso-Galicia et al., 1999). While 20-HETE is predominantly a vasoconstrictor, it dilates bovine coronary arteries and pulmonary arteries (Pratt et al., 1998; Yuan et al., 1996) an action that is blocked by subsequent addition of indomethacin (Campbell et al., 1998). This suggests that a COX metabolite of 20-HETE is responsible for the vasodilating effects in these vascular beds.
Vasoactive agents such as Angiotensin II (Ang II), and norepinephrine (NE) activate phospholipase A to release AA from membrane phospholipids pools. 20-HETE blocks the large-conductance, calcium activated potassium (K$_{Ca}$) channel in the vascular smooth muscle cells, which results in membrane depolarization and enhances Ca$^{2+}$ influx through the L-type, voltage gated Ca$^{2+}$ channels.

1.5.3.2. Renal actions of 20-HETE

20-HETE plays an important role in the regulation of tubular reabsorption of sodium. In the proximal tubules (PT), 20-HETE inhibits renal Na$^+$-K$^+$-ATPase activity
(Schwartzman et al., 1985) while in the thick ascending limb of loop of Henle (TALH) 20-HETE regulates chloride transport by inhibiting \( \text{Na}^+ - \text{K}^+ - 2\text{Cl}^- \) cotransport (Carroll et al., 1991). 20-HETE blocks the \( \text{K}^+ \) channel in the apical membrane of TALH cells and limits \( \text{K}^+ \) availability for transport via the \( \text{Na}^+ - \text{K}^+ - 2\text{Cl}^- \) cotransporter (Wang & Lu, 1995). Thus, by blocking \( \text{K}^+ \) recycling, 20-HETE inhibits sodium reabsorption and subsequently facilitates natriuresis. Thus, the effect of 20-HETE in PT and TALH is natriuretic.

Figure 6. Renal actions of 20-HETE

20-HETE inhibits sodium reabsorption in the PT by inhibiting \( \text{Na}^+ - \text{K}^+ - \text{ATPase} \) by activating protein kinase C (PKC). In the TALH, 20-HETE inhibits sodium transport by blocking a 70-pS \( \text{K}^+ \) channel in the apical membrane. Blockade of this channel limits \( \text{K}^+ \) availability for transport via the \( \text{Na}^+ - \text{K}^+ - 2\text{Cl}^- \) cotransporter.
1.5.4. Appearance and detection of 20-HETE in urine

Several studies have shown the formation of 20-HETE in the kidney of rats. Schwartzman et al., (1991) confirmed that 20-HETE is excreted in the urine of SHRs. Urinary 20-HETE levels probably reflect renal synthesis (Sacerdoti et al., 1997). It is important to note that in humans, 20-HETE undergoes glucuronide conjugation and is excreted as a conjugate of glucuronic acid. The fact that 1-ABT failed to completely inhibit urinary excretion of 20-HETE led to a speculation that 20-HETE might be blood borne and undergo glomerular filtration and tubular excretion (Maier et al., 2000). There is no evidence for this. Urinary 20-HETE levels can be detected fairly accurately using a HPLC. The detection limit is 1 pg. While collecting urine for 20-HETE analysis from rats in metabolic cages it is important to withdraw food from the rats as rat chow is known to bind to 20-HETE. Also urine should be collected in dry ice as 20-HETE is unstable in urine.

1.6. Role of 20-HETE in hypertension

There is overwhelming evidence that the renal production of 20-HETE is altered in many experimental and genetic models of hypertension.

1.6.1. Experimental models of hypertension

1.6.1.1. DOCA-salt hypertension

The DOCA salt model of hypertension represents the hypertension in humans that is attributed to increased mineralocorticoid activity and possibly, elevated endothelin levels. Endothelin stimulates the release of 20-HETE from the kidney and 20-HETE contributes
to the renal vasoconstrictor and natriuretic actions of endothelin (Oyekan et al., 1997 & 1998). The renal excretion of 20-HETE is elevated fourfold in rats treated with DOCA (deoxycorticosterone acetate and salt (1% NaCl in the drinking water) (Oyekan et al., 1999). However, one study reported that the production of 20-HETE is reduced rather than elevated in DOCA-salt hypertensive mice (Honeck et al., 2000).

1.6.1.2. Ang II-induced hypertension

ANG II, stimulates the renal synthesis of 20-HETE in rats (Croft et al., 2000) and rabbits (Carroll et al., 1996). Inhibiting the synthesis of 20-HETE attenuates the vasoconstrictor actions of ANG II in isolated renal arteries in vitro suggesting that 20-HETE contributes to the vasoconstrictor actions of Ang II. Moreover, in vivo studies suggest that 20-HETE also contributes to the pressor response to Ang II infusion (Alonso-Galicia et al., 2000). Further, chronic blockade of the renal formation of EETs and 20-HETE with 1-ABT attenuated the development of hypertension in ANG II infused rats.

1.6.2. Genetic models of hypertension.

1.6.2.1. Spontaneously hypertensive rat

The expression of CYP 450 4A2 gene is elevated in the kidney of spontaneously hypertensive rat (SHR) (Iwai and Inagami, 1991). The production of 20-HETE is elevated while EET formation is reduced in the kidney of SHR (Sacerdoti et al., 1988). Moreover, induction of hemeoxygenase with chronic administration of SnCl₂ or inhibiting 20-HETE formation with 1-ABT prevents the development of hypertension in SHR (Escalante et al., 1991).
1.6.2.2. Dahl salt-sensitive rat

When Dahl S rats are fed a high-salt diet, mean arterial pressure typically increases by 20 mm Hg within 24 hours and continues to rise to a plateau of 170 mm Hg or higher, around 2 weeks (Rapp et al., 1985; Greene et al., 1990; Roman, 1987). The initial rise in arterial pressure appears to be triggered by sodium retention. The animal gains about 7% in body weight; plasma volume and cardiac output increase significantly (Greene et al., 1990). Later, cardiac output returns to normal values, and the hypertension is maintained by increased peripheral vascular resistance (Simchon et al., 1991). The pressure-natriuresis relationship remains shifted to the right so that the kidney requires a higher perfusion pressure to excrete the same amount of sodium as normotensive rats (Roman, 1988 & 1991). The renal production of 20-HETE is reduced in the outer medulla but not in the cortex of the Dahl SS/Jr rats relative to the Dahl SR/Jr (salt-resistant) rats and other normotensive strains (Ma et al., 1994; Makita et al., 1994). 20-HETE regulates Cl− transport in the TALH of the rat suggesting that a reduction in the formation of 20-HETE might contributes to the elevation in Cl− transport in Dahl S rats (Escalante, 1991 & 1994). Induction of the expression of CYP 4A protein in the kidney of Dahl S rats with fibrates normalizes the pressure-natriuretic relationship (Alonso-Galicia et al., 1998), and prevents the development of hypertension (Roman et al., 1991, 1994 & Wilson et al., 1998). It also reduces the degree of proteinuria and glomerular injury (Wilson et al., 1998). Thus elevation in renal 20-HETE production improves renal function and prevents the development of hypertension in the Dahl S rats.

1.7. Nitric oxide
F. Murad, R.F. Furchgott and L.J. Ignarro won the 1998 Nobel Prize for their characterization of endothelium derived relaxing factor as nitric oxide. NO is synthesized by the enzyme nitric oxide synthase (NOS). NOS catalyses the synthesis of NO from the guanidino nitrogen atoms of the amino acid L-arginine.

NO induces vasorelaxation via cGMP dependent and cGMP independent mechanisms. NO increases the production of soluble cGMP, a second messenger through activation of guanylyl cyclase (Murad, 1986). cGMP further decreases inositoltriphosphate (IP₃), by decreasing phospholipase C activity. cGMP also causes decrease in [Ca²⁺], and decreased activity of myosin light chain kinase. Decreased [Ca²⁺], subsequently causes hyperpolarization of the VSMC acting directly on the Ca²⁺ dependent K⁺ channel (Murad 1999). However, NO also appears to act independent of cGMP by inhibiting the production of 20-HETE (Sun et al., 2000).

### 1.8. NO – 20-HETE Interaction

The interaction between NO and 20-HETE varies in different vascular beds. In renal microsomes, sodium nitroprusside, a NO donor, inhibited the formation of 20-HETE in a dose dependent but cGMP independent manner (Sun et al., 1998). NO also inhibits heme-containing proteins including NOS and CYP. Inhibition of the formation of 20-HETE attenuated the pressor effects of L-NAME suggesting that NO tonically inhibits 20-HETE formation (Oyekan et al., 1998b). However, NO contributes to the vasodilatory action of 20-HETE in pulmonary arteries (Yu et al., 2002). 20-HETE evokes vasodilation in the pulmonary artery in an endothelium dependent and COX – dependent manner (Jacobs et al., 1999).
1.8.1. NO and endothelial dysfunction:

Endothelial dysfunction is defined as loss of normal vasodilating, anti-thrombotic and anti inflammatory function of endothelium. Hypertension is one of many factors that can cause endothelial dysfunction, although the relation between blood pressure and any measure of endothelial dysfunction is not close (Vaziri 2004). Numerous studies suggest that loss of the biological activity of nitric oxide and/or its biosynthesis is the central mechanism responsible for endothelial dysfunction (Katusic, 2001). The exact mechanisms responsible for endothelial dysfunction in arteries exposed to chronic hypertension are not completely understood, but may involve chemical antagonism between superoxide anions and nitric oxide and functional antagonism of nitric oxide-mediated vasodilatation due to release of endothelium-derived contracting factor(s) (Katusic & Shepherd, 1991; Vanhoutte, 1996).

1.9. Hypotheses and predictions of the working hypothesis

1.9.1. Hypotheses

1. Young, but not adult S-D rats will retain sodium and increase blood pressure during a high salt challenge; they are unable to increase renal 20-HETE synthesis.

2. Adult rats will remain normotensive during a high salt challenge; they are able to increase renal 20-HETE synthesis.

3. Clofibrate will render the young animals salt resistant.
1.9.2. Working hypothesis and predictions

1. Young S-D rats are more salt-sensitive than adult ones

A prediction of this hypothesis is that the young rats will retain sodium and develop hypertension when given a high salt intake, while the adult ones will remain normotensive. Moreover, clofibrate will prevent the development of hypertension in the young animals.

2. 20-HETE aids in the excretion of sodium

Adult rats will increase the urinary excretion of 20-HETE when given a high salt challenge while the young ones will not be able to increase urinary 20-HETE excretion levels. Moreover, clofibrate will increase urinary 20-HETE levels in the saline treated young rats.

3. Salt-sensitivity will cause decreased plasma NO\textsubscript{x} and increase oxidative stress

We predicted that the young saline treated rats will demonstrate decreased plasma NO\textsubscript{x} and generate more superoxide anions from the aorta when compared to the adult rats.

4. CYP 4A mRNA expression in salt-sensitivity

We predicted that the young salt-sensitive, but not the adult salt-resistant animals, will have unchanged or even decreased expressions of CYP 450 4A mRNA in the renal outer medulla/renal cortex, while clofibrate will upregulate CYP expression.
2.0 Materials and Methods

2.1 Materials

2.1.1 Animals

Four week old male S-D rats weighing about 150-175 g and one year old male S-D rats were obtained from Charles River (St. Constant QC). The animals were housed in a dedicated animal facility with a 12 hour light/dark cycle, given low Na+ chow (0.4% by weight of NaCl) to eat and tap water to drink ad libitum. The animals were housed singly in metabolic cages throughout the course of the experiment.

2.1.2 Chemicals

The chemicals used in the experiments and their sources are listed below:

*Chemicals used in functional studies:*

Clofibrate was obtained from Sigma Aldrich Canada Ltd (Oakville, ON)

NaCl and Na₂CO₃ were obtained from BDH (Toronto, ON)

Clofibrate was dissolved in 20mmol Na₂CO₃.

*Chemicals used in RNA Extraction:*

Absolute alcohol and β - mercaptoethanol were obtained from EMD Biosciences and Sigma – Aldrich (Oakville, ON) respectively.

*Chemicals used in HPLC assay:*

20-HETE was purchased from Cayman chemical company (Ann Arbor, MI)

N,N-diisopropylethylamine (catalyst) and 2-(2,3 – naphthalimino) ethyl trifluoromethanesulfonate (dye) were purchased from Sigma-Aldrich (Oakville, ON) and Molecular Probe (Eugene, OR) respectively.
Methanol, acetic acid, THF, ethyl acetate were purchased from EMD Biosciences.

2.1.3 Equipment

2.1.3.1 Apparatus for measuring blood pressure:
The apparatus consists of a restrainer to hold the rat which was placed on a thermal blanket maintained at 37°C. The BP monitor (Harvard apparatus, Boston, MA) was connected to a tail cuff plethysmograph and a sphygmomanometer. The SBP was recorded using the Power Lab Data Acquisition System (AD Instruments Pvt. Ltd., Sydney, Australia).

2.1.3.2 Apparatus for measuring 20-HETE:
A High Pressure Liquid Chromatography (HPLC) system was used to measure urinary 20-HETE levels. It consisted of the following components:

1. A Hitachi L-7100 pump
2. A Hitachi L-7200 auto sampler
3. A C-18 reverse phase HPLC column
4. A Hitachi L-7485 fluorescence detector
5. A Hitachi interface D-7000

The Hitachi L-7100 pump, pumps the mobile phase at a constant flow rate. A fixed set volume of the sample is automatically withdrawn from the sample vial by the autosampler and is injected into the mobile phase of the liquid chromatograph. The C-18 reverse phase HPLC column (4.6X250 mm, Waters Symmetry, Milford, MA) separates CYP metabolites including 20-HETE using a mobile phase. The mobile phase contains
81% methanol, 0.1% acetic acid. The Hitachi L-7485 fluorescence detector reads the sample set at excitation wavelength of 259 nm and an emission wavelength of 394 nm which is converted to a digital signal by a Hitachi Interface D-7000. The 20-HETE peak was compared to that of an internal standard. Data was processed using a Hitachi model D-7000 Chromatography data sampler software.

2.1.3.3 Apparatus for superoxide anion measurement: Luminometer
A TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA) measures the light energy (photon) emitted from the interaction of lucigenin with O$_2$. Lucigenin in the buffer that contains the aortic tissue gains an electron from the O$_2$ and emits light. The luminometer reads and integrates the arbitrary units of light emitted over a period of 30 seconds. The readings are taken over a continuous period of five minutes and are averaged.

2.1.3.4 Apparatus for measuring plasma nitrates
Anthos Lit plate reader to read total plasma nitrite, and nitrate

2.1.3.5 Apparatus for measuring protein
Bausch and Lomb Spectrophotometer to read protein concentrations

2.2 Methods
2.2.1 Systolic blood pressure
SBP was measured 5 times a day every two days for three weeks, after acclimatizing the animals to the warmed restrainer using a previously calibrated tail cuff plethysmograph. The values for the day were averaged.

2.2.2 Assay for 20-HETE

Urine was centrifuged at 1000g for 10 minutes. 250 µl of the clear supernatant was acidified to pH 3.0 with 10% HCl. Fifty ng of an internal standard, WIT-002, was added to the urine. Then, three volumes of ethyl acetate were added and centrifuged at 1000g for 10 minutes. The supernatant was removed carefully without disturbing the lipid layer and dried under nitrogen. It was resuspended in 200 µl of acetonitrile and vortexed for 20 seconds and 800 µl of filtered water was added. The above mixture was again vortexed for 20 seconds before applying it, in total, to the Sep-Pak.

Preparation of Sep-Pak-Vac:

A C 18 Sep-Pak-Vac 1 cc 50 mg (Waters) was used. It was prewashed with 1 ml H2O, 4 ml ethyl acetate, followed by 1 ml H2O. The sample was applied with a light vacuum.

The Sep-Pak was washed twice with 1 ml of 30% acetonitrile. The sample was eluted with 400 µl of 90% acetonitrile. 900 µl of filtered water was added to eluent.

Again a C18 Sep-Pak – Vac was prewashed as described before. The sample was applied with a light vacuum, which was eluted with 500 µl ethyl acetate. It was dried under nitrogen.

10 µl of the fluorescent dye N,N-Diisopropylethylamine was added and vortexed for 20 seconds. It was let to stand at room temperature for 30 minutes. After that it was dried under nitrogen and resuspended in 400 ul acetonitrile and vortexed for 20 seconds.
600 μl of filtered water was added to the sample. This mixture was applied to a prewashed Sep-Pak-Vac to remove any unreacted fluor. The Sep-Pak was again washed 6 times with 1 ml of 50% acetonitrile. The sample was eluted with 500 μl ethylacetate and dried under nitrogen. This was resuspended in 100 μl of methanol and transferred to a amber glass tube with a microinsert fitted with a cap with septa.

50 μl of the sample was injected onto a Symmetry C 18 column with a mobile phase of 82% Methanol and 0.1% acetic acid for 95 minutes. Fluorescence was recorded using an inline fluorescence detector – excitation 259 nm and emission 394 nm. The amount of the 20-HETE in the sample was determined by comparing the area of the 20-HETE peak to that of the internal standard. For each assay, a standard curve was constructed using known amounts of authentic 20-HETE. The total 20-HETE excretion rate was calculated by multiplying the concentration times the 24 hour urine output.

The percent recovery of 20-HETE in our experiment was about 90%.

### 2.2.3 Collection and measurement of urine

The rats were placed in metabolic cages (Nalgene, Rochester, N.Y.) which efficiently separated urine from feces. Urine was collected in precooled plastic tubes placed on dry ice below the metabolic cages. Volume was determined gravimetrically.

### 2.2.4 Measurement of urine sodium

Urine Na⁺ was measured by flame spectrophotometry in the hospital chemistry lab.
2.2.5 Estimation of sodium balance

Na⁺ balance is defined as the difference between total Na⁺ intake and Na⁺ output in a given time period. The weight of the food consumed and the volume of fluid intake determined total Na⁺ intake. The food and fluid contained known concentrations of Na⁺. The total Na⁺ output was determined using the concentration of Na⁺ in the urine and total urine output.

\[
\text{Na⁺ balance} = \text{Na⁺ intake} - \text{Na⁺ output}
\]

2.2.6 Determination of urinary protein

Urine protein concentration was measured using the Bio-Rad reagent, which uses bovine albumin as standard. A Bausch and Lomb spectrophotometer was used to quantify absorbance at 595 nm. Proteinuria was estimated as total urinary protein excretion in a 24-hour period.

2.2.7 Measurement of superoxide anion

The aortic rings were prepared from freshly dissected aorta. They were rinsed with Kreb’s buffer and then incubated in test tubes containing 2 ml of HEPES buffer. The composition of the HEPES buffer was HEPES 20 mM, NaCl 119 mM, KCl 4.6 mM, MgSO₄ 2 mM, Na₂HPO₄ 0.15 mM, KH₂PO₄ 0.39 mM, NaHCO₃ 5 mM, glucose 5.6 mM, CaCl₂·2H₂O 1.6 mM. After incubating the aortic rings for 20 min they were then transferred to test tubes containing 500 μl of HEPES buffer (pH 7.4) at 37°C to which lucigenin (5 μM) was added. Rings were incubated with lucigenin for 15 min. The tubes were transferred to the luminometer. The arbitrary units of light emitted were read over a
preset period of 30 seconds and were integrated by the luminometer. 8 repeated measurements over a period of 5 minutes were done and averaged (Reading 1). Tiron (10 mM), a cell permeable nonenzymatic scavenger of O$_2^-$, was then added to the buffer in which aortic rings were incubated to quench the O$_2^-$ dependent chemiluminescence. After incubating for 15 minutes, 8 readings were repeated over 5 minutes and the values were averaged (Reading 2). The difference between readings 1 and 2 was used to calculate O$_2^-$ production. The tiron quenchable O$_2^-$ production by the aortic rings were calculated using this formula.

Superoxide anion = \[
\frac{2 \times (\text{Reading 1} - \text{Reading 2}) \times 1000}{\text{weight of aortic ring in mg}}
\]

### 2.2.8 Measurement of plasma total nitrites and nitrates

Blood was collected in pre-chilled EDTA tubes and was centrifuged at 1000g for 10 minutes. The plasma was collected and stored at -80 °C until plasma nitrates were assayed. Plasma nitrates were assayed using a commercial NO$_x$ colorimetric assay kit. Ultrafiltration of plasma was done using a 10 kDa molecular weight cut-off filter (Pall Corporation, Ann Arbor, Michigan) using a centrifuge. Ultrafiltration reduces background absorbance due to the presence of hemoglobin and improves color formation by the Griess reagents. The measurement of total plasma nitrates involves a two step process. The first step is the conversion of nitrate to nitrite utilizing nitrate reductase. The second step is the addition of the Griess reagents which convert nitrite into a deep purple azo compound. Photometric measurement of the absorbance due to this azo chromophore accurately determines nitrite concentration.
2.2.9 Real – Time PCR for CYP 450 4A

2.2.9.1 Isolation of RNA

2.2.9.1.1 Separation of renal cortex and outer medulla

Rats were anesthetized with thiopentone sodium (100 mg/kg i.p), and the abdominal aorta was cannulated with a polyethylene PE-50 catheter below the left renal artery. The blood flow to the left kidney was briefly interrupted, and the kidney was flushed with 10 ml of cold dissection solution (4°C). The dissection solution consisted of 135 mM NaCl, 3 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 2 mM KH₂PO₄, 5.5 mM glucose, 5 mM L-alanine, and 10 mM HEPES (pH 7.4). After flushing, the left kidney was rapidly removed, and placed in 20 ml of fresh dissection solution and oxygenated with 95%O₂ and 5% CO₂ (Ito and Roman, 1998). The cortex and outer medulla were carefully dissected out and placed in RNAlater® (Qiagen). The outer medulla was dissected out at the cortico-medullary junction and care was taken to avoid contamination with the cortex and the inner white medulla.

2.2.9.1.2 Stabilization of RNA

The renal cortical and medullary tissues were cut into slices less than 0.5 cm thick. The tissues were submerged in 10 volumes (10 µl/1 mg of tissue) of RNAlater® (Qiagen). The samples were incubated overnight at 4°C and were stored at -80°C until extraction of RNA.
2.2.9.1.3 Extraction of RNA

The tissues were thawed and approximately 30 mg of the tissue was used for extraction of RNA. The entire extraction procedure was carried out at 25°C.

The tissues were homogenized for 40 sec using a Rotor-stator homogenizer in 600 µl of buffer RLT placed in RNase free tube.

The tissue lysate was centrifuged for 3 min at maximum speed in a microcentrifuge.

The supernatant was carefully transferred to a new microcentrifuge tube by pipetting.

This supernatant was used in subsequent steps.

1 volume (600 µl) of 70% ethanol was added to the cleared lysate, and was mixed immediately by pipetting.

700 µl of the sample was applied to the RNeasy® mini column placed in a 2 ml collection tube and was centrifuged for 15 sec at maximum speed in a microcentrifuge.

The flow through was discarded.

700 µl of Buffer RW1 was added to the RNeasy column and centrifuged at maximum speed to wash the column. The flow through and the collection tube were discarded.

The RNeasy column was transferred into a new 2 ml collection tube. 500 µl of Buffer RPE was pipetted out into the RNeasy column and was centrifuged at maximum speed to wash the column. The flow through was discarded.

Again, 500 µl of Buffer RPE was added to the RNeasy column. The column was centrifuged for 2 min at maximum speed to dry the RNeasy silica-gel membrane.

The RNeasy column was placed in a new 1.5 ml collection tube for elution. 30- 50 µl of RNase-free water was directly added on to the RNeasy silica-gel membrane and centrifuged for 1 min at maximum speed to elute.
The extracted RNA was shipped by courier to the laboratory of Dr. Richard B. Kim, Professor, Division of Clinical Pharmacology, Vanderbilt University, Nashville, TN.

2.2.9.2 Quantification of RNA

RNA quantity was assessed using a spectrophotometer at 260 nm wavelength.

2.2.9.3 Qualitative analysis of RNA

A DNA digest was performed to remove any genomic DNA.

15 μl RNA stock sample was mixed gently with 0.1 volume of Turbo DNase Buffer and 1μl Turbo DNase (Ambion® The RNA Company) and briefly centrifuged. The mixture was incubated for 30 min at 37°C.

After incubation, 2µl of suspended DNase Inactivation Reagent was added and further incubated for 2 min at room temperature, mixing gently 2-3 times during incubation.

The samples were centrifuged at 10,000 g for 90 sec.

The supernatant was transferred to a new tube.

RNA quality was assessed by agarose gel electrophoresis which showed strong and clear band for 18S and 28S RNA.

2.2.9.4 Synthesis of cDNA

cDNA synthesis was performed using 1μg or 0.5μg total-RNA with the TaqMan® Reverse Transcription Reagents kit (Applied Biosystems). All the steps were carried out on ice.
1000 ng of RNA was used per reaction

100 µl final volume of reaction
10 ng/µl final concentration of RNA in the reaction

<table>
<thead>
<tr>
<th>Components of RT Reaction Mix</th>
<th>1X Fold reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Taqman® RT Buffer</td>
<td>1.00 µl</td>
</tr>
<tr>
<td>25 mM MgCl2</td>
<td>2.20 µl</td>
</tr>
<tr>
<td>dNTP Mix 2.5 mM</td>
<td>2.00 µl</td>
</tr>
<tr>
<td>Random Hexamere 50 µM</td>
<td>0.50 µl</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>Multiscribe Reverse Transcriptase 50U/ µl</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>Final volume of the RT reaction mix</td>
<td>6.15 µl</td>
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</table>

**Example Reaction:**

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<tr>
<th>Sample</th>
<th>RNA sample Concentration (ng/µl)</th>
<th>RNA sample Volume (µl)</th>
<th>DEPC- H₂O (µl)</th>
<th>RT-reaction Mix volume (µl)</th>
<th>Total volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>29.6</td>
<td>33.8</td>
<td>4.7</td>
<td>61.5</td>
<td>100</td>
</tr>
</tbody>
</table>

Incubation 10 minutes at 25°C

Incubation 30 minutes at 48°C

Inactivation 5 minutes at 95°C

Hold at 4°C

**2.2.9.5 Determination of CYP 450 4 A expression using RT-PCR**

Real-Time PCR for all genes was carried out with the Primers mentioned below using a iCycler IQ™ Real_time Detection System (Biorad).

Primers:
rat CYP4A1 Forward (FW)  TCCAGGCATTGTCAGAGAACT
rat CYP4A1 Reverse (RV)  TAAATGGAGAGTGACTTGGATA
rat CYP4A2 FW            AGATCCAAAGCCCTTATCAATC
rat 4A2, 4A3 common RV   TGATCCTGGTCATCAAGCTTC
PCR reactions were carried out in a 50μl reaction using 200nM final Primer concentration and iQ SYBR® Green Supermix (Bio-Rad)

RT-PCR Conditions:

<table>
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<tr>
<th>Final reaction volume (μl)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Sample volume</td>
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</tr>
<tr>
<td>Concentration of forward primer solution (μM)</td>
<td>100</td>
</tr>
<tr>
<td>Concentration of reverse primer solution</td>
<td>100</td>
</tr>
<tr>
<td>Final primer concentration (FW and RV) nM</td>
<td>200</td>
</tr>
</tbody>
</table>

RT-PCR Reaction Mix:

<table>
<thead>
<tr>
<th>1x Fold Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
</tr>
<tr>
<td>Reverse primer</td>
</tr>
<tr>
<td>iQ™ SYBR® Green Supermix</td>
</tr>
<tr>
<td>Water</td>
</tr>
</tbody>
</table>

The cycle conditions are described below, 40 cycles of PCR were performed for all CYP4A genes, and 34 cycles were performed for 18S genes.
Protocol for CYP 4A genes:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Repeats</th>
<th>Step</th>
<th>Ramp Rate</th>
<th>Dwell Time</th>
<th>Hold</th>
<th>Set-point</th>
<th>Melt Curve</th>
<th>+ Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>MAX</td>
<td>3 min</td>
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<td>95.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>1</td>
<td>MAX</td>
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<td></td>
<td>95.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>2</td>
<td>MAX</td>
<td>30 sec</td>
<td></td>
<td>65.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>MAX</td>
<td>1 min</td>
<td></td>
<td>95.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>80</td>
<td>1</td>
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<tr>
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<td>1</td>
<td>1</td>
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</tbody>
</table>

Protocol for 18S genes:

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<th>Repeats</th>
<th>Step</th>
<th>Ramp Rate</th>
<th>Dwell Time</th>
<th>Hold</th>
<th>Set-point</th>
<th>Melt Curve</th>
<th>+ Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>MAX</td>
<td>3 min</td>
<td></td>
<td>95.0</td>
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<tr>
<td>2</td>
<td>34</td>
<td>1</td>
<td>MAX</td>
<td>15 sec</td>
<td></td>
<td>95.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>1</td>
<td>MAX</td>
<td>1 min</td>
<td></td>
<td>65.0</td>
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<tr>
<td>4</td>
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<td>1</td>
<td>MAX</td>
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<tr>
<td>6</td>
<td>1</td>
<td>1</td>
<td>MAX</td>
<td>10 sec</td>
<td>yes</td>
<td>55.0</td>
<td>yes</td>
<td>0.5</td>
</tr>
</tbody>
</table>

For all genes a melting curve analysis was carried out, temperature increases by 0.5°C for 10s starting at 55°C. This temperature increase was performed 80 times.

PCR standards: purified PCR products with known copy numbers were used.

A calibration curve was performed to calculate the number of copies of the samples using iCycler™ iQ Optical system software version 3.0a (Biorad)

2.3. Experimental Protocol

2.3.1. Treatment Groups

The rats were housed singly in metabolic cages in temperature – controlled rooms in a 12hour light/dark cycle for 5 days before the beginning of the actual experimental
The experiments were started at 5 weeks of age for the young rats and at 52 weeks of age for the adult rats. The young rats were randomly assigned to 4 subgroups and, for drinking water, were given either tapwater to which 20 mmol Na₂CO₃ had been added (control) or normal saline (0.9% NaCl) to which 20 mmol Na₂CO₃ had been added. Half the number of animals in each group received clofibrate (80 mg/day) (Roman et al., 1993). This dose was that used in previous experiments. The concentration of clofibrate was changed so that each animal took in about 80 mg/day. Blood pressure was measured using tail cuff apparatus every day other for three weeks.

2.3.2. Determination of urinary 20-HETE and sodium balance

For estimation of 20-HETE and sodium balance, food, but not water, was withdrawn from the rats and urine was collected for a continuous period of 24 hours in tubes placed on dry ice. Urine was collected on days 1, 7, 14 and 21. The urine volume was estimated over this 24 hour period.

2.3.3. Estimation of proteinuria

Proteinuria was estimated from a single 24 hour urine collection at the end of three weeks.

2.3.4. Determination of vascular superoxide anion production

After three weeks of treatment the thoracic aorta was removed under anesthesia and the aortic superoxide anion generation was estimated using lucigenin chemiluminescence.
2.3.5. Determination of plasma total nitrites/nitrates

After three weeks of treatment, the rats were anesthetized with thiopentone sodium (100 mg/kg i.p.) and blood was withdrawn from the carotid artery and collected in pre-chilled EDTA tubes and centrifuged to obtain plasma from which plasma total nitrite/nitrates were assayed.

2.3.6. Determination of CYP 450 4A mRNA expression in kidney

After three weeks of treatment, the animals were anesthetized and the left kidney was removed after flushing with ice cold dissection solution from which the renal cortex and outer medulla were separated and stored for extraction of RNA and subsequent real-time RT-PCR analysis of CYP 4A mRNA expression.

2.4. Statistical analyses

Data were transferred to a Prism 3.0 spreadsheet and analyzed with the embedded program. The data from the young group of rats were analyzed using one-way ANOVA followed by a Bonferroni’s post-hoc test while unpaired students’ t-test (2-tailed) was applied to the data from the adult group. P<0.05 or lower was deemed statistically significant.
3.0 Results

3.1. Effect of salt and clofibrate on systolic blood pressure

Saline treatment did not affect SBP in the adult rats (fig 7a). The control group of rats had an average BP of $117 \pm 2$ mmHg and the saline group had an average BP of $117 \pm 1$ mmHg at the end of three weeks. There was no significant change in BP over a three week period in either group.

The young rats had an average BP of about $100$ mmHg at the beginning of the treatment period i.e., at the age of 5 weeks (fig 7b). There was a gradual increase in BP in the control group over a three week period so that the SBP at the end of a three weeks was $115 \pm 2$ mmHg. Saline induced an increase in BP, so that the SBP at three weeks was about $134 \pm 2$ mmHg ($p<0.001$ vs control). The BP in the saline treated group was significantly higher compared to the control group after day 13. Clofibrate did not change BP in the control group whose average BP at three weeks was $114 \pm 2$ mmHg. Clofibrate not only prevented the increase in BP in the saline treated rats but decreased BP to values lower than the control group at the end of three weeks. The average BP in clofibrate treated, saline drinking animals was $102 \pm 2$ mmHg ($p<0.001$ vs control and saline).

3.3. Effect of salt and clofibrate intake on urine volume

There was a gradual increase in the urine volume in both vehicle and saline treated groups over three weeks (fig 9a). Saline treatment induced a significant increase in the average urinary volume as early as day 1. The average daily urine volume, over three
weeks, in the saline treated group (65.8 ± 2.2 ml/d) was significantly higher when compared to the control group (27.25 ± 1.5 ml/d) p<0.001.

There was a gradual increase in urine volume in the control group of young rats, over the three week treatment period (fig 9b). Saline treatment induced a modest increase in urine volume in the young rats which became significantly higher than the control group (48.14 ± 7, 20.5 ± 2.5 ml/d, p<0.05) at the end of a three week treatment period. Clofibrate did not affect urine volume in the control group. However, clofibrate induced a 4 fold increase in urine volume in the saline treated group at three weeks (83.83 ± 5.2 ml/d p<0.001 vs. control group).

3.4. Effect of salt and clofibrate on sodium balance

Vehicle treated adult rats had a positive Na⁺ balance of 2.7 ± 0.2 mmol/d at the three week time period. Saline treated animals retained 5.2 ± 0.5 mmol (p<0.01) during the same day (fig 10a).

Control rats had an average Na⁺ balance of about 2.63 ± 1 mmol/d at the end of the three week treatment period, which was unaffected by clofibrate (3.9 ± 1 mmol/d) (fig 10b). In contrast, saline treated rats retained 22.9 ± 3.2 mmol/d at the end of the three week treatment period while clofibrate treated saline drinking animals retained only 8 ± 2.75 mmol/d (p<0.05 vs. saline group).
3.2. Effect of salt and clofibrate on urinary 20-HETE levels

Compared to the control group, saline induced an increase in urinary 20-HETE excretion in adult rats, which was significantly higher after two weeks (fig 8a). At the end of the three week period, the control group had an average urinary 20-HETE excretion of $322 \pm 6$ ng/d and the saline treated group had an average urinary 20-HETE excretion of $425 \pm 8$ ng/d ($p<0.001$) (fig 8a).

Urinary 20-HETE levels in the control group of young rats were in general higher than that in the adult rats (fig 8b). However, there was a gradual decrease in the urinary 20-HETE levels in the control group over a three week period. As expected, clofibrate induced a significant increase in the urinary 20-HETE levels, which was significantly higher even at the end of the first week. The average urinary 20-HETE levels at the end of the three week treatment period was $938 \pm 16$ ng/d. Clofibrate induced an increase in the urinary 20-HETE levels in the control group at the end of a two week treatment period. The average urinary 20-HETE levels at the three week treatment period was $552 \pm 10$ ng/d. In contrast to the adult rats, saline treatment did not increase, and indeed, even decreased urinary 20-HETE excretion when compared to the control group ($452 \pm 16$, $384 \pm 17$ $p<0.05$).

3.5.1. Effect of salt and clofibrate on aortic superoxide anion production

$O_2^-$ levels were measured in at least four aortic rings from each animal after three weeks of treatment in the adult rats. Saline treatment did not affect the aortic $O_2^-$ production in the adult group of rats ($1107.5 \pm 159$, $1247 \pm 265$ milliunits/mg/min) (fig 11a).
O$_2^-$ levels were measured in at least four aortic rings from each animal after three weeks of treatment in the young rats. Saline treatment induced a significant increase in the aortic O$_2^-$ production in the young rats when compared to the control group (2388.8 ± 40.4 vs. 1617.3 ± 82.8 milliunits/mg/min p<0.001) which was significantly reduced by clofibrate treatment (1378.8 ± 64.2 p<0.001 milliunits/mg/min vs. saline) (fig 11b). Clofibrate did not have a significant effect on aortic O$_2^-$ production in the control group.

3.6.1. Effect of salt and clofibrate on plasma nitrite/nitrate levels

Saline treatment did not affect the plasma total nitrite/nitrate levels in the adult rats at the end of a three week treatment period (6.5± 1, 5.4 ± 0.3 μM) (fig 12a).

In contrast to the adult rats, saline treatment induced a significant decrease in the plasma NO$_x$ levels (4.2 ± 0.5 vs. 9.6 ± 0.7 μM p<0.001) at the end of a three week treatment period. Clofibrate did not affect plasma NO$_x$ levels in either group (fig 12b).

3.7.1 Effect of salt and clofibrate on urinary protein levels

Saline treatment induced an increase in the urinary protein levels in the adult rats (799 ± 35 vs. 455 ± 18 μg/ d, p<0.01) (fig 13a).

Saline treatment induced an almost four fold increase in the urinary protein levels in the young rats (1936 ± 214 vs. 597 ± 82 μg/ 24h, p<0.001) which was reduced by clofibrate treatment (1258 ± 77 μg/ 24h vs. saline, p<0.05) (fig 13b). However, clofibrate did not significantly affect urinary protein levels in the control group (722 ± 37 μg/ 24h).
3.8. Effect of salt and clofibrate on CYP 4A mRNA expression in renal cortex

Saline treatment induced an increase in the renal cortical mRNA expressions of CYP 4A1, 4A2 and 4A3 (fig 14a Panels A, B, C). CYP 4A1 was induced almost 5 fold while CYP 4A2 and 4A3 were increased by 2 fold. mRNA expression of CYP 4A8 in the renal cortex did not increase significantly(fig 14a Panel D).

As expected, clofibrate induced the expressions of CYP 4A1, 4A2 and 4A3 significantly when compared to both the control and the saline treated groups (fig 14b panels A, B, C). Clofibrate treatment induced a ~ 4 fold increase in the CYP 4A1 and a ~ 2 fold increase in the CYP 4A2 and 4A3 when compared to the saline treated group. Clofibrate did not significantly affect the mRNA expressions in the control group. In contrast to the adult rats, saline treatment induced a significant decrease in the mRNA expressions of CYP 4A1 and 4A3 (Panels A & C). The mRNA expression of CYP 4A8 was unaffected by salt and clofibrate treatment (fig 14b Panel D).

3.9.1. Effect of salt and clofibrate on CYP 4A mRNA expression in renal outer medulla

Saline treatment did not significantly affect the expressions of any of the four CYP 4A mRNA expressions in the renal outer medulla (fig 15a).

In contrast to the adult rats, saline treatment induced a significant decrease in the mRNA expressions of CYP 4A2 and CYP 4A3 (fig 15b Panels B, D). Clofibrate did not have a significant effect on the mRNA expressions of both the control and the saline treated groups.
Figure 7a. Effect of salt intake on systolic blood pressure in adult rats

Each value is an average of 7 animals per group at each time point. Values are expressed as mean ± SEM at each time point. Statistical analysis was performed using unpaired t-test. There was no significant difference between two groups at any time point throughout the three week treatment period.
Figure 7b. Effect of salt and clofibrate on systolic blood pressure in young rats

Each value is an average of at least 6 animals per group at each time point. Values are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni's post-hoc test. * p<0.05, *** p<0.001 compared to control group; ψ p<0.05, ψψ p<0.01, ψψψψ p<0.001 compared to saline group; # p<0.001 compared to control group at the same time point.
Figure 8a. Effect of salt intake on urine volume in adult rats

Each value is an average of 7 animals per group at each time point. Values are expressed as mean ± SEM at each time point. Statistical analysis was performed using unpaired t test. ** p<0.01, *** p<0.001 compared to age matched control at the same time point.
Figure 8b. Effect of salt and clofibrate on urine volume in young rats

Each value represents the average urine volume from at least 6 rats per group at each time point. Values are represented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni's post-hoc test. * p<0.05, *** p<0.001 compared to the control group at the same time point. ψψψ p<0.001 compared to saline group at the same time point.
Figure 9a. Effect of salt intake on sodium balance in adult rats

Each value is an average of 7 animals per group at each time point. Values are expressed as mean ± SEM at each time point. Statistical analysis was performed using unpaired t test. ** p<0.01 compared to age matched controls at the same time point.
Figure 9b. Effect of salt and clofibrate on sodium balance in young rats

Each value represents the average Na⁺ balance from at least 6 rats per group at each time point. Values are mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni's post-hoc test. ** p<0.01 compared to the control group. ψ p<0.05, ψψ p<0.01 compared to the saline group at the same time point.
Control (n=7)

Saline (n=7)

Figure legends on next page
Figure 10a. Effect of salt intake on urinary 20-HETE levels in adult rats

Figure represents the average urinary 20-HETE excretion levels from 7 animals per group on days 1, 7, 14 and 21. Values are expressed as mean ± SEM. Statistical analysis was performed using unpaired t test. * p<0.05, *** p<0.001 compared with age matched control at the same time point.
Figure 10b. Effect of salt and clofibrate on urinary 20-HETE levels in young rats

Figure represents the average urinary 20-HETE excretion levels from at least 6 animals per group on days 1, 7, 14 & 21. Values are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni’s post-hoc test. * p<0.05 compared to control group; # p<0.05 compared to control group; ψψψ p<0.001 compared to saline group at the same time point.
Figure 11a. Effect of salt intake on superoxide anion production in adult rats after three weeks.

Each value is an average of 4 animals per group. Values are expressed as mean ± SEM. Statistical analysis was performed using unpaired t test. There was no significant difference between the two groups.
Figure 11b. Effect of salt and clofibrate on superoxide anion production in young rats after three weeks

Each value represents the average $O_2^-$ production from 4 rats per group. Values are represented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni’s post-hoc test. *** p<0.001 compared to the control group, ψψψ p<0.001 compared to saline group.
Figure 12a. Effect of salt intake on plasma total nitrite/nitrate in adult rats after three weeks

Each value is an average of at least 5 animals per group. Values are expressed as mean ± SEM. Statistical analysis was performed using unpaired t test. There was no significant difference between the two groups.
Figure 12b. Effect of salt and clofibrate on plasma total nitrite/nitrate levels in young rats after three weeks

Each value represents the average plasma NO\textsubscript{x} levels from 6 rats per group. Values are represented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni's post-hoc test. *** p<0.001 compared to the control group.
Figure 13a. Effect of salt intake on urinary protein excretion in adult rats after three weeks

Figure represents the average 24 hour urinary protein excretion from 7 animals per group on day 21 of treatment. Values are expressed as mean ± SEM. Statistical analysis was performed using unpaired t test. ** p<0.01 compared to age matched control.
Figure 13b. Effect of salt and clofibrate on urinary protein excretion in young rats after three weeks

Each value represents the average urinary protein excretion over a 24 hour period on day 21 in the young rats. Values are mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni’s post-hoc test. *** p<0.001 compared to the control group. ψ p<0.05 compared to the saline group.
Figure 14a. Effect of salt intake on CYP 4A mRNA expression in renal cortex of adult rats.

Panels A, B, C & D represent the average mRNA expressions of CYP 4A1, 4A2, 4A3 & 4A8 from three animals per group on day 21 of treatment. Values are expressed as mean ± SEM. Statistical analysis was performed using unpaired t test. * p<0.05, ** p<0.01, *** p<0.001 compared to age matched control.
Figure 14b. Effect of salt and clofibrate on CYP 4A mRNA expression in renal cortex of young rats

Panels A, B, C, and D represent the average mRNA expressions of CYP 4A1, 4A2, 4A3 and 4A8 respectively on day 21 of treatment from three rats per group. Values are mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni's post-hoc test. * p<0.05, ** p<0.01 compared to the control group. ψ p<0.05, ψψψ p<0.001 compared to the saline treated group.
Figure 15a. Effect of salt intake on CYP 4A mRNA expression in renal outer medulla of adult rats.

Panels A, B, C & D represent the average mRNA expressions of CYP 4A1, 4A2, 4A3 & 4A8 respectively from three animals per group on day 21 of treatment. Values are expressed as mean ± SEM. Statistical analysis was performed using unpaired t test. There was no significant difference between the two groups.
Figure 15b. Effect of salt and clofibrate on CYP 4A mRNA expression in renal outer medulla of young rats

Panels A, B, C, and D represent the average mRNA expressions of CYP 4A1, 4A2, 4A3 and 4A8 respectively on day 21 of treatment from three rats per group. Values are mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni’s post-hoc test. * p<0.05 compared to the control group.
4.0 Discussion

In the present study, we demonstrate that high salt intake (given in the form of 0.9% NaCl in the drinking container) induces an increase in BP in young (5 weeks) Sprague-Dawley rats. This was accompanied by increased Na\(^+\) retention, decreased urinary 20-HETE excretion and decreased mRNA expressions of CYP4A in the renal cortex and outer medulla. On the other hand, high salt intake in the adult (52 weeks) rats did not increase BP, and increased urinary excretion of 20-HETE and increased the mRNA expression of CYP4A in the renal cortex. Clofibrate, a lipid lowering agent and a PPAR-\(\alpha\) agonist induced CYP4A mRNA expression in the renal cortex, increased urinary 20-HETE levels and prevented the development of hypertension in the young rats on a high salt intake.

4.1 Effect of high salt intake and clofibrate on systolic blood pressure:

In our study the young, but not the adult S-D rats developed hypertension when given saline to drink. Typically our animals drank 50-80 ml of 0.9% NaCl daily so that their Na\(^+\) intake was about 50 mmol per Kg. This is in the same range as that which causes severe hypertension in Dahl salt-sensitive rats fed an 8% NaCl diet (Wilson et al., 1998). However, there have been inconsistencies regarding the effect of high salt intake on BP in S-D rats. Sofola et al (2002) observed an increase of 34 mm Hg with 8% NaCl diet in weanling rats. A recent study has observed only 12 mm Hg increase in SBP with 4%
NaCl diet for a period of two weeks in S-D rats weighing 175 - 200 g (likely about 8-10 weeks of age)(Williams et al., 2004). Another study in male Wistar rats of about 4 and 12 weeks of age found similar increases in both age groups (Zicha et al., 2003). However, S-D rats weighing about 300-350 g (about 14-16 weeks of age) given a 8% NaCl diet for two weeks did not develop hypertension (Hoagland et al., 2003). Makita et al also found no increases in BP when S-D rats weighing 300-350 g were given 8% NaCl for 5 weeks. These inconsistencies can be attributed to the age of the animal, the NaCl content of the diet and the duration of treatment. However, in our study all the young animals on a high salt intake developed hypertension and served as our salt-sensitive model, while the BP did not increase in any of our adult rats. They represented the salt-resistant animal.

Clofibrate not only prevented the development of hypertension in the saline treated young rats but also lowered BP even below control values. Our study confirms the anti-hypertensive effect of clofibrate, that prevented the development of hypertension in the Dahl SS/Jr on a 8% NaCl diet (Roman et al., 1993). Fenofibrate a related drug also prevented the development of hypertension in the Dahl SS/Jr rats on a high salt diet (Wilson et al 1998; Shatara et al., 2000) and to a certain extent in the young SHR-SP (Shatara et al., 2000). An interesting finding was that clofibrate had no effect on BP in rats drinking tap water (with 20mmol Na₂CO₃) but actually lowered BP in saline treated rats. This suggests that salt somehow changed the response to clofibrate: a “salt-specific” hypotensive property of clofibrate.
4.2 Effect of high salt intake and clofibrate on sodium balance and urine volume

A high salt intake resulted in an increased retention of Na\(^+\) in the young rats but not in the adult rats compared to their age matched controls. This Na\(^+\) retention should lead to increased cardiac output and increased blood pressure (Roman RJ and Cowley A, 1996). Our findings are consistent with the studies by Wilson et al (1998) in which Dahl SS/Jr rats had a positive cumulative Na\(^+\) balance which was prevented by fenofibrate treatment. In our study, clofibrate decreased Na\(^+\) balance in the saline treated young rats but not in age matched control demonstrating its specificity on salt intake. A high salt intake resulted in an increase in the urine output both in the young and the adult rats. Clofibrate induced a significant increase in urine volume in the saline treated young rats. This data is consistent with previous reports that fenofibrate increased urine volume in the Dahl SS/Jr rats on a high salt diet (Shatara et al., 2000).

4.3 Effect of high salt intake and clofibrate on urinary 20-HETE levels:

Our urine 20-HETE assay relies on HPLC with fluorometric detection. It is felt to be relatively specific but may measure metabolites other than 20-HETE. As the metabolic fate of 20-HETE in the kidney is presently unknown, we accept that our measurement reflects changes in 20-HETE and possibly metabolites of 20-HETE.

The young rats when given a high salt diet demonstrated a decreased urinary excretion of 20-HETE when compared to the age matched control whereas the adult rats showed increased urinary 20-HETE excretion when given a high salt diet when compared to the age matched counterpart. This is one of the few studies where the urinary excretion of 20-
HETE in response to a high salt intake has been measured. Consistent with our data on the adult group of rats, Hoagland et al have also shown a 20% increase in urinary 20-HETE excretion in S-D rats weighing about 300-350 g (about 14-16 weeks of age) when given a high salt diet. Importantly, these rats did not develop an increase in BP.

However, Oyekan et al have demonstrated significantly decreased ω-hydroxylase activity, measured by the metabolism of AA, in the renal cortex but not in the renal outer medulla of S-D rats weighing about 270 g, given 2% NaCl in drinking water. The authors failed to measure BP in these rats.

Several studies indicate an altered renal metabolism of AA and formation of 20-HETE in the Dahl SS rats.

There is a decreased formation of 20-HETE in both the renal cortex and outer medulla of Dahl SS rats on a low-salt diet (Ma et al., 1994), without a significant difference in the CYP4A protein expression between the Dahl SS/Jr and Dahl SR/Jr.

There are no studies of the change in CYP4A expression in Dahl SS rats when fed a high salt diet, but as mentioned above, such animals do not increase 20-HETE formation in kidney homogenates. A decreased renal metabolism of AA in the outer medulla but not in the renal cortex of the Dahl SS/Jr rats when compared to Lewis rats given a 1% NaCl diet has been reported (Stec et al., 1996). Moreover, the authors found a significant decrease in the CYP4A protein expression in both the renal cortex and the outer medulla of the Dahl SS rats when compared to the Lewis rats. These studies were confirmed by Ito et al.
who demonstrated that the CYP4A protein expression is significantly decreased in the TALH when compared to the Dahl SR/JR rats (Ito et al., 1998).

We attribute salt sensitivity of BP in our group of young rats to decreased renal production of 20-HETE. These findings are consistent with previous studies in which chronic blockade of the renal formation of 20-HETE with an intrarenal infusion of 17-ODYA induced salt-sensitive hypertension in Lewis rats (Stec et al., 1997) and that inhibitors of 20-HETE promote salt-sensitive hypertension in S-D rats (Hoagland et al., 2003).

Clofibrate induced a significant increase in the urinary excretion of 20-HETE in the saline treated young rats. The origin and significance of 20-HETE in the urine are unknown. 20-HETE does not circulate in blood (Roman RJ Personal communication), and is produced in several cell types in the kidney, including VSMC and tubular cells. CYP 4A is expressed in virtually all areas of the kidney save the inner medulla (Ito et al., 1998). We therefore submit that urinary 20-HETE reflects renal synthesis. Sacerdoti et al., (1997) are of similar opinion. Of interest, in our studies, is the apparent lack of correlation between urine volume and 20-HETE excretion. Both young and adult rats increased urine volume in response to increasing salt intake, but only the older rats increased 20-HETE excretion.

Our study confirms previous observations that clofibrate induced a significant increase in the renal \( \omega \)-hydroxylase activity in the renal cortex and the renal outer medulla of the Dahl SS rats (Roman et al., 1993). The CYP 450 4A \( \omega \)-hydroxylase is the enzyme that forms 20-HETE from AA. Hence an increased renal \( \omega \)-hydroxylase activity represents
an increased renal synthesis of 20-HETE. A related drug, fenofibrate increased the renal ω-hydroxylase activity and CYP 450 4A protein expression only in the renal cortex (Wilson et al., 1998). The inner medulla makes little 20-HETE.

4.4 Effect of high salt intake and clofibrate on plasma nitrates

In our study we determined total plasma NOx that are stable metabolites of nitric oxide, as a measure of NO formation. NO is rapidly oxidized to nitrite and subsequently to nitrate by oxygenated hemoglobin, molecular O2 and by O2⁻ anions as well and is excreted as such in the urine (Fujiwara et al., 2000). Thus, measurement of NOx appears to represent the NO formation to a reasonable extent. However, this method is not without disadvantages. Nitrates may arise from sources other than the metabolism of NO. In mammalian cells, however, NO is formed from the guanidino nitrogen atoms of the amino acid L-arginine, and this is the only known route by which these nitrogen atoms may be incorporated into nitrate. Therefore, the measurement of total NOx may be a specific indicator of total body NO synthesis. However, these results should be interpreted with caution as dietary nitrate may contribute to plasma NOx. In our study, all rats received a similar diet so that, dietary nitrate may not be a confounding factor.

In the present study a high salt intake induced a significant decrease in the plasma NOx in the young but not in the adult rats. Clofibrate did not change plasma NOx in either the control or the saline treated animals.

Our results are in agreement with a previous study reporting decreased NOx in the perfusate of isolated kidney from a SS/Jr when compared to a SR/Jr (Hayakawa et al.,
On the other hand, one study has reported S-D rats given a 1% NaCl in drinking water for 2 weeks show an increased serum total NO\textsubscript{x} levels and an increased urinary excretion of total NO\textsubscript{x} (Shultz et al., 1993).

A number of mechanisms by which a high salt intake can lead to a decreased formation of NO have been proposed. Salt loading attenuates the conversion of L-arginine to NO in the renal vascular endothelium in salt-sensitive patients with essential hypertension (Higashi et al., 1996). It has been well documented that the L-arginine – NO pathway is impaired in salt-sensitive experimental hypertension by reduced bioavailability of endothelial NO (Luscher et al., 1987; Chen et al., 1991; Chen et al., 1993; Miyata et al., 1998; Chen et al., 1998). The suppressant effect of high salt intake on plasma NO\textsubscript{x} concentration could occur by several mechanisms, including altered transport of L-arginine through the endothelial membrane, decreased activity of NOS, and an increased breakdown or urinary excretion of NO (Singh et al., 1995). ADMA an endogenous NOS inhibitor that also inhibits L-arginine uptake into endothelial cells is increased in the plasma of humans after a high salt intake (Fujiwara et al., 2000). These mechanisms are plausible explanations for our results. However, our study was not designed to address the mechanism by which a high salt intake decreased plasma NO\textsubscript{x} levels. Decreased NO\textsubscript{x} would be expected to lead to endothelial dysfunction, but would not necessarily cause hypertension.
4.5 Effect of high salt intake and clofibrate on superoxide anion formation

A high salt intake induced an increased formation of aortic $O_2^-$ formation in the young but not the adult rats which was prevented by clofibrate. An increased level of $O_2^-$ anion formation indicates a high level of oxidative stress. A 4% NaCl diet induced an increased aortic $O_2^-$ formation in S-D rats of about 8 -12 weeks of age (Zhu et al., 2004). An increased $O_2^-$ formation has been associated with other hypertensive models and has been observed in the mesenteric arterioles of SHR (Suzuki et al., 1995 & 1998), abdominal aorta of SHR-SP (Kerr et al., 1999), and microvessels of the mesentery of Dahl SS rats (Swei et al., 1997 & 1999). This is the first study in which clofibrate has been shown to prevent salt induced increase in $O_2^-$ formation in the S-D rats. Fenofibrate has been shown to decrease vascular $O_2^-$ generation in DOCA salt model of hypertension (Iglarz et al., 2003). Since fenofibrate, a related drug decreased vascular $O_2^-$ formation in the DOCA-salt model of hypertension without an effect on blood pressure, it is reasonable to speculate that the $O_2^-$ lowering effect of clofibrate could be a direct effect of the drug than being secondary to a reduction in BP. Moreover, we have observed that even though L-NAME, a NOS inhibitor caused fulminant hypertension it almost completely inhibited the generation of $O_2^-$ and the levels were even lower than the control group (unpublished observation). Hence neither the generation nor the inhibition of $O_2^-$ formation appears to be a direct effect of changes in BP.

4.6 Effect of high salt intake and clofibrate on proteinuria

In the present study, a high salt intake induced a significant increase in urinary protein excretion, a marker of renal damage, in the young rats compared to the age matched
counterpart. Even though a high salt intake increased proteinuria in the adult rats it were not as high as the saline treated young rats. Clofibrate prevented saline induced proteinuria in the young rats. However, in one study a high salt intake did not induce significant protein excretion in the Dahl SS/Jr rats (Shatara et al., 2000). But fenofibrate decreased saline induced proteinuria in the Dahl SS/Jr rat fed a high salt diet (Wilson et al., 1998) and the SHR-SP (Shatara et al., 2000).

4.7 Effect of high salt intake and clofibrate on CYP 4A mRNA expression

We showed, for the first time, that CYP4A mRNA expression in the kidney increases after a high salt intake.

As expected, clofibrate induced the expressions of CYP 4A1, 4A2 and 4A3 in the renal cortex of saline treated rats. This data is consistent with previous observations that clofibrate (Roman et al., 1993) and fenofibrate (Wilson et al., 1998) induced renal cortical ω-hydroxylase activity in Dahl SS rats on a high salt diet. Clofibrate also induced the renal outer medullary ω-hydroxylase activity in the Dahl SS rats on a high salt diet (Roman et al., 1993). However in our study clofibrate induced only the renal cortical CYP4A mRNA expression. Clofibrate did not induce the renal CYP4A mRNA expression in control group of rats, suggesting a specific action of clofibrate on saline treated animals.

A high salt intake induced significant decrease in the mRNA expressions of CYP4A2 and 4A3 in the renal outer medulla and CYP A1 and 4A3 in the renal cortex of the young
rats which are in agreement with previous reports that Dahl SS rats had decreased CYP 4504A protein expression in both the renal cortex and the renal outer medulla (Stec et al., 1996). In the same study Stec et al observed that the renal cortical ω-hydroxylase activity in the Dahl SS rats on a high salt diet were unaffected.

In contrast to the young rats, a high salt diet increased renal cortical mRNA expressions of CYP 4A1, 4A2 and 4A3 without affecting the renal outer medullary CYP expression. Previous studies have shown that clofibrate (Roman et al., 1993) and fenofibrate (Wilson et al., 1998) prevented the development of salt sensitive hypertension in Dahl SS rats by increasing renal synthesis of 20-HETE. However, whether this salt sensitivity of BP could have resulted from decreased CYP4504A mRNA expression that forms 20-HETE and whether the actions of clofibrate are mediated through upregulation of CYP4504A gene was unknown. Moreover, whether salt can induce the expression of CYP4504A and aid in the excretion of salt in the salt resistant model has not been explored.

Thus, this study investigated the effect of a high salt intake on the development of high BP and its relation to CYP 450 4A gene using the young S-D rats as our salt-sensitive model and the adult S-D rats as our salt-resistant model.

When given a high salt diet the young rats retain Na⁺ and develop hypertension. The decreased ability to excrete Na⁺ is probably due to the fact that these rats fail to increase renal synthesis of 20-HETE when challenged with a high salt diet. 20-HETE inhibits Na⁺K⁺ATPase activity and Na⁺ reabsorption in the proximal tubule (Satoh et al., 1993;
Ribeiro et al., 1994). 20-HETE also inhibits chloride transport in the TALH (Escalante et al., 1991) by blocking a 70pS K⁺ channel (Wang et al., 1995) that is required for the recycling of K⁺ and the operation of the Na⁺K⁺2Cl⁻ cotransporter. Thus a deficiency in the renal formation of 20-HETE plays a critical role in elevating loop Cl⁻ transport, resetting the pressure natriuresis and the development of hypertension in Dahl SS rats (Ito et al., 1999; Zou et al., 1996).

A decreased renal synthesis of 20-HETE appears to be due to the inhibition of CYP4A mRNA in the renal cortex and the outer medulla by a high salt intake in the young rats. An increase in BP after a high salt intake could also be attributed to decreased formation of NO as suggested by decreased plasma NOx. The mechanisms by which salt could decrease NO production have been addressed in previous sections. In our study we have observed an increased vascular O₂⁻ formation in saline treated young rats as opposed to adult rats. O₂⁻ scavenges NO to form ONOO⁻ a reaction that is three times faster than the catalysis of O₂⁻ by SOD. Hence another possible reason for a decreased plasma NOx levels could be due to increased formation of O₂⁻.

Thus a high salt intake affects renal 20-HETE synthesis at the transcription level. In contrast, the adult rats when given a high salt intake do not retain much Na⁺ and do not develop hypertension. This is probably because a high salt intake increases the mRNA expression of CYP4A and increases the renal synthesis of 20-HETE, which aids in the excretion of salt. Also, a high salt intake did not affect the production of NO. Clofibrate, a PPAR-α agonist and lipid lowering agent induces the mRNA expression of CYP4A in
both the renal cortex and the outer medulla of salt fed young rats, and likely, increases renal 20-HETE synthesis, decreases Na\(^+\) balance and prevents the development of hypertension. However, the mechanism by which it reduced BP in the saline treated young rats even lower than the age matched control group has not been addressed in this study.

It is important to observe that after 2 weeks urinary excretion of 20-HETE reached maximum levels (with no further increases beyond this level at three weeks). At three weeks there was a dramatic increase in urine volume accompanied by a sudden decrease in Na\(^+\) balance. Previous studies have failed to confirm whether the increase in renal synthesis of 20-HETE is accompanied by an increase in urine output and Na\(^+\) excretion (Roman et al., 1993; Wilson et al., 1998; Shatara et al., 2000) despite several studies confirming that 20-HETE prevents Na\(^+\) reabsorption in the kidney. It is also important to observe that clofibrate failed to completely prevent Na\(^+\) retention after three weeks. However, based on the decreasing trend in Na\(^+\) balance in saline treated rats it is reasonable to speculate that prolonged treatment with clofibrate might completely prevent Na\(^+\) retention.

One might ask how clofibrate could lower BP in saline treated rats even lower than controls despite not completely eliminating Na\(^+\). This is likely possible that some of these actions could be attributed to the reduction of vascular O\(_2\)\(^-\) levels and other mechanisms through induction of epoxygenase activity which we will address below. Clofibrate that decreased vascular O\(_2\)\(^-\) levels failed to restore plasma NO\(_x\) levels back to normal. This
could possibly have been due to an increased urinary excretion of NO\textsubscript{x} as a result of diuresis induced by clofibrate. Measurement of urinary NO\textsubscript{x} levels could resolve this issue.

Clofibrate can induce the renal CYP 2C23 (Muller et al., 2004) which produce EETs. Fenofibrate, a related drug has been shown to induce renal CYP2C23 activity and prevent Ang II induced renal injury (Muller et al., 2004). Clofibrate has been shown to induce CYP mono/epoxygenases in the endothelial cells in vitro and evoke membrane hyperpolarization (Hoebel et al., 1998). Epoxygenases form EETs which are known to dilate renal arteries of rats and rabbits (Carroll et al., 1993; Imig et al., 1996; Zou et al., 1996). Several studies have suggested that EETs are potent vasodilators (Gebremedhin et al., 1992; Imig et al., 1996). EETs hyperpolarize VSMC (Gebremedhin et al., 1992; Hecker et al., 1994; Campbell et al., 1996; Fukao et al., 1997 and Eckman et al., 1998) by increasing the open state probability of K\textsubscript{Ca} channels (Campbell et., 1996 & 1999) which is thought to attenuate Ca\textsuperscript{2+} entry via voltage-sensitive channels.

Clofibrate also reduced the degree of renal damage as evidenced by it ability to decrease saline induced proteinuria in the young rats. This confirms previous studies where fenofibrate prevented saline induced proteinuria, reduced the degree of mesangial expansion and glomerulosclerosis (Wilson et al., 1998). Whether this is due to lower blood pressure or some other effect of clofibrate is unknown.
A significant increase in Na\(^+\) balance well before the development of hypertension in the saline treated young rats would suggest an inability to excrete Na\(^+\) as a primary causative factor in the development of hypertension.

In summary, the present results indicate that a high salt intake resulted in the downregulation of the CYP4A gene in the kidney of the young rats decreasing the renal synthesis of 20-HETE leading to Na\(^+\) retention and development of hypertension. For the first time we demonstrate that, a high salt intake in the adult rats upregulated the CYP 4A gene increased the renal synthesis of 20-HETE, prevented Na\(^+\) retention and development of hypertension. Furthermore, clofibrate a CYP4A inducer, as expected induced CYP 4A gene, increased renal synthesis of 20-HETE prevented Na\(^+\) retention, and decreased BP in the saline treated young rats. However, the mechanism by which salt induced CYP 4A gene has not been addressed in this study.

In conclusion, we demonstrate for the first time that a high salt intake induces the expression of CYP4A gene whose product 20-HETE aids in the excretion of salt.
5.0 Limitations

We measured urine 20-HETE with a HPLC fluorometric assay. While such assays are said to be relatively specific, we cannot categorically say our assay is 100% specific for 20-HETE. Using a very specific assay such as LC-mass spectrometry could confirm that 20-HETE is indeed increased in urine. Specific agonists or antagonists of 20-HETE would add further certainty. If a specific 20-HETE antagonist or ω-hydroxylase inhibitor prevented the natriuretic response to salt or clofibrate, then either 20-HETE or a metabolite of 20-HETE would be implicated in the natriuresis. Unfortunately, to our knowledge, such compounds do not exist.

6.0 Speculations and future directions

Like all research projects, this one leaves unanswered questions and prompts speculations. The following include some of these as well as suggestions for further research.

What is the mechanism by which high salt intake increases CYP4A and 20-HETE in older animals?
Our studies shed no light on this question. When faced with a chronically elevated salt intake, the organism undergoes several changes. The renin-angiotensin-aldosterone system, the vasopressin system and the sympathetic nervous system are suppressed, while the natriuretic peptide system is activated. The former group could tonically inhibit CYP4A expression, or the natriuretic peptides could increase it.

The renin-angiotensin system is maximally suppressed (at least in humans) at relatively low salt intakes: in the range of 120 mmol of sodium per day. The average intake in western societies is about 175 mmol per day. Thus, the very high salt intake we used would certainly suppress renin, but perhaps not too much more than a regular salt intake. Furthermore, all land animals are highly adapted to conserve sodium; a sodium wasting state would lead to decreased cardiac output and hypotension. If the renin-angiotensin system were tonically inhibiting CYP4A, a powerful sodium excreting mechanism, then turning it off would lead to profound natriuresis. Renin is turned off at “normal” sodium intake, but intense natriuresis does not occur. Similar arguments can be made for vasopressin and the sympathetic nervous system.

It seems more likely that one or more natriuretic peptides might act to enhance natriuresis via CYP4A. These peptides are produced in response to volume overload and act to correct it through excreting sodium. One logical experiment would be to treat animals on a normal salt intake with a natriuretic peptide, and measure CYP4A expression and 20-HETE. If the natriuretic peptide enhanced CYP4A at normal (or low) sodium intake, our hypothesis would be supported.
Why didn’t the young rats increase their 20-HETE synthesis and excrete the sodium load?

Young rats did not increase CYP4A expression and 20-HETE production with salt, but did when exposed to clofibrate. This suggests that the gene for CYP4A is fully developed and capable of responding to a PPARα agonist. It further suggests that salt and clofibrate increase 20-HETE by different mechanisms. If a natriuretic peptide is responsible for CYP4A enhancement in older animals, perhaps levels do not rise as much in younger animals or the response to the peptide is blunted.

Also, atrial extracts from young S-D rats (about 3-5 weeks) show decreased levels of atrial natriuretic peptide when compared to the adult rats. Moreover, an immature atrial natriuretic peptide system in the young rats would to a reasonable extent support our speculation that the effect of natriuretic peptide is mediated through either formation or release of 20-HETE. Hence low levels of ANP or an immature natriuretic system in the young rats could be a reason for decreased 20-HETE formation and subsequently decreased urinary excretion of 20-HETE when challenged with a high salt diet.

Which CYP4A metabolite causes natriuresis?

20-HETE is known to cause natriuresis. Clofibrate induces renal synthesis of 20-HETE in the proximal tubule and the TALH. It also induces CYP 2C in the kidney as well as vascular endothelium which synthesized EETs. In particular clofibrate induces CYP 2C23 in the kidney and increases EET formation. EETs inhibit sodium transport by...
inhibiting the translocation of Na\(^+\)/H\(^+\) exchanger to the apical membrane of the proximal tubule. Sodium chloride has been shown to increase the formation of EETs in the kidney of S-D rats. In our study, we have not measured the formation of EETs.

**Why was the blood pressure in clofibrate/saline treated animals lower than that in clofibrate/water treated ones?**

As shown in the results section, clofibrate and saline treated young animals had lower blood pressure than clofibrate and vehicle treated ones. This was an important experiment as it argues against a non-specific hypotensive effect of clofibrate. Moreover, in experiments not detailed here, we showed that single doses of clofibrate, given intraperitoneally, do not lower blood pressure in anesthetized rats. We feel that clofibrate, while enhancing CYP4A activity, may also increase CYP2C activity in vascular endothelial cells. This leads to the increased production of arachidonic acid metabolites other than 20-HETE, such as 11,12-epoxyeicosatrienoic acid and other EETs. In contrast to 20-HETE, some EETs evoke membrane hyperpolarization by opening potassium channels and cause vasodilation. We are currently measuring CYP2C mRNA expression in our rat kidney specimens.

**Is there a teleologic reason why clofibrate (or other PPAR\(\alpha\) agonists) should cause sodium excretion?**

The PPAR\(\alpha\) receptor binds a number of ligands including medium and long chain fatty acids. It then leads to increased expression of a number of genes involved in fatty acid metabolism. In general, these enzymes breakdown triglycerides (eg lipoprotein lipase)
and metabolize fatty acids by omega hydroxylation oxidation. In most cases these omega hydroxy fatty acids are further oxidized to carbon dioxide and water and supply considerable energy to cells in the process.

Rats, like many land animals are opportunistic feeders, and may on occasion consume excessive calories. The PPARα system may be a defense against caloric over consumption. One could speculate that a large fatty acid or triglyceride intake, might be associated with a large, and possibly toxic, sodium load. In that case, a natriuretic mechanism, linked to PPARα would confer an advantage.

**Do fibrates cause natriuresis or lower blood pressure in humans?**

To our knowledge none of the large fibrate trials have specifically asked this question. The Helsinki Heart Study, the VA-HIT study and the DAIS study were all designed to examine the effect of fibrates on lipid fractions and cardiovascular outcomes rather than blood pressure. In all, people with hypertension were included but not analysed as to differences in BP.

Given the sensitivity of Ambulatory Blood Pressure Monitoring in detecting even a small change in blood pressure, a simple trial of fibrates in people with hypertension seems worthwhile.
Could 20-HETE excretion (or change in excretion) be a marker for salt sensitive hypertension in humans?

It seems plausible that 20-HETE should increase in humans, as well as rats, in response to a very high salt intake. It is well known that many people can tolerate high salt diet, while others develop hypertension. It is very tempting to speculate that people who can increase 20-HETE would be salt-resistant.
7.0 References


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