ORIGINAL ARTICLE

Chronic clofibrate administration prevents saline-induced endothelial dysfunction and oxidative stress in young Sprague-Dawley rats

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Abstract

Purpose: High salt intake causes hypertension and endothelial dysfunction in young Sprague-Dawley rats. Clofibrate (clof) prevents this salt induced hypertension. We asked whether clof can prevent salt-induced endothelial dysfunction, and if so, its mechanism. We also questioned whether high salt intake can induce endothelial dysfunction without hypertension in older animals.

Methods: Young (Y, 5 weeks) and old (O, 53 weeks) male Sprague-Dawley rats were given either vehicle (Con, 20 mM Na₂CO₃) or 0.9% NaCl (Sal) to drink for three weeks. Some young rats received clof (80 mg/d) in their drinking fluid. After three weeks, we measured mean arterial pressure (MAP), endothelial function, by comparing hypotensive responses to acetylcholine (ACh, endothelium dependent) and sodium nitroprusside (SNP, endothelium independent), plasma total nitrite+nitrate levels (PNOx), by the Griess reaction, and aortic superoxide production by lucigenin chemiluminescence.

Results: Carotid artery MAP did not change in O. Sal-Y developed hypertension: 133±3 vs. 114±2 mmHg, $P<0.001$, which was prevented by clof: 105±2 mmHg. ACh induced a similar dose dependent hypotensive response in Con-O and Sal-O that was inhibited by L-NAME (100mg/kg *i.v.*). Responses to ACh were blunted in Sal-Y but not in Con-Y. Further, L-NAME inhibited ACh responses only in Con-Y. The response to SNP was similar in all animals. Importantly, the ACh-induced hypotensive re-

sponse was potentiated in clof+Sal-Y, an effect which was attenuated by blocking calcium-activated potassium channels (K_{Ca}) with a combination of apamin (50 ug/kg *i.v.*) + charybdotoxin (50 ug/kg *i.v.*), but not by L-NAME. PNOx was reduced in Sal-Y compared to Con-Y (2.09±0.26 vs. 4.8±0.35 μ M, $P<0.001$), but not in Sal-O. Aortic superoxide production was higher ($P<0.001$) in Sal-Y (2388±40 milliunits/mg/min) than Sal-O (1107±159 milliunits/mg/min), but was reduced by clof (1378±64 milliunits/mg/min; $P<0.001$).

Conclusions: High salt intake increases oxidative stress in young animals, leading to impaired nitric oxide activity and endothelial dysfunction. Clofibrate prevents endothelial dysfunction partly through reduced O₂⁻ formation but mainly *via* selective activation of K_{Ca} channels. Older animals are resistant to both salt induced hypertension and oxidative stress.

High salt intake can result in the development of hypertension in some humans¹ and animals.^{2,3} High salt intake has also been reported to cause endothelial dysfunction, with or without a change in blood pressure.⁴⁻⁶ Endothelial dysfunction is characterized by reduced endothelium dependent vascular relaxation.⁷ Endothelium dependent vascular relaxa-

tion is due to several mediators produced by the endothelium including nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin. In salt-induced hypertension, NO mediated vascular relaxation is reduced and is partially compensated by EDHF.⁸ EDHF is a non-NO, non-prostanoid endothelium-derived hyperpolarizing factor that persists after effective inhibition of nitric oxide synthase and cyclo-oxygenase pathways.^{9,10} EDHF dependent vasorelaxation is mediated through calcium-activated potassium channels (K_{Ca}).¹¹ Several substances may comprise EDHF, including epoxyeicosatrienoic acid (EET), anandamide, hydrogen peroxide, and potassium ion.¹²

Clofibrate, a lipid lowering fibrate, has been shown to prevent the development of salt-sensitive hypertension.^{13,14} We have recently shown that clofibrate acutely reverses saline induced endothelial dysfunction in young Sprague-Dawley rats *via* activation of K_{Ca} .¹⁵ Moreover, in our previous study, we showed that only young Sprague-Dawley rats develop hypertension on a high salt intake. We attributed this difference to the inability of the young rats to increase cytochrome P4504A expression and renal 20-HETE, an endogenous natriuretic.¹⁴ Therefore, these animals retained sodium and developed hypertension. In the present study we aimed to determine if salt caused endothelial dysfunction, without hypertension, in older rats. We also asked if long term clofibrate administration would not only prevent hypertension, but also endothelial dysfunction, in young animals, and if so, the mechanism(s) involved.

Materials and methods

Materials

Acetylcholine (ACh), sodium nitroprusside (SNP), N^G -nitro-L-arginine methyl ester hydrochloride (L-NAME), clofibrate, apamin (Apa), lucigenin, tiron were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON,) and charybdotoxin (ChTx) from Ana Spec (San Jose, CA). The nitrite/nitrate colorimetric

assay kit was purchased from Cayman Chemical (Ann Arbor, MI). ACh, SNP and L-NAME were dissolved in normal saline (0.9%NaCl) (sodium chloride), clofibrate was dissolved in 20mM Na_2CO_3 and Apa and ChTx were dissolved in 0.1% bovine serum albumin (1 mg/ml) and further diluted in normal saline before administration.

Animals

Four week old (Y) and 52 week old (O) male Sprague-Dawley rats were purchased from Charles River (St. Constant, QC, Canada) and housed in our dedicated animal facility in a temperature-controlled room with a 12-h light/dark cycle. The experimental protocol was approved by the University Committee on Animal Care and Supplies at the University of Saskatchewan according to the guidelines of the Canadian Council for Animal Care.

Assessment of endothelial function in vivo

Hypotensive responses to ACh (0.02, 0.06, 0.2, 0.6, 2 $\mu\text{g kg}^{-1}$ i.v.) and SNP (0.06, 0.2, 0.6 $\mu\text{g kg}^{-1}$ i.v.) were obtained before, and 45 min after L-NAME (100 mg kg^{-1} i.v.),¹⁶ and 20 min after a combination of Apa (50 $\mu\text{g kg}^{-1}$ i.v.) and ChTx (50 $\mu\text{g kg}^{-1}$ i.v.).¹⁷ Dose response curves to ACh and SNP vs. MAP were then obtained. Some animals from each group were given indomethacin 10 mg/Kg i.v, 30 minutes prior to ACh. Pretreatment with indomethacin did not affect MAP or endothelium dependent relaxation. The baseline mean arterial pressures were different in the Con and Sal groups of young rats. Therefore, the hypotensive responses to ACh and SNP were calculated as percent fall in MAP with respect to the baseline MAP before each response. We, and others, have previously shown that the dose dependent, percent fall in MAP after ACh is unrelated to the baseline MAP.^{15,18,19} The responses to ACh and SNP were determined in a minimum of five rats per group and the values are expressed as mean $\pm$ SEM. In some animals undergoing the same treatments, 2 ml of blood was drawn through

the carotid artery under anesthesia into vacutainer tubes containing ethylenediaminetetracetic acid. After centrifuging at 4°C, plasma was stored at -80°C until assayed for plasma nitrites and nitrates (PNO_x), a marker of nitric oxide production.²⁰

Superoxide anion measurement using lucigenin derived chemiluminescence

From the same animals used for measuring PNO_x, the aorta was harvested and tiron quenchable superoxide anion was measured using lucigenin (5µM) as described previously.²¹

Experimental protocol

At 53 weeks of age, O (*n*=28) were randomly assigned to either 20 mM Na₂CO₃, vehicle for clofibrate (control, Con; *n*=14) or 0.9% NaCl (Sal, *n*=14) in their drinking water. At 5 weeks of age, Y (*n*=63) were divided into two groups. They received either vehicle (*n*=29) or 0.9% NaCl (*n*=34). Some Y from both groups also received clofibrate (*n*=27) at a dose of 80 mg daily in their drinking fluid.^{13,14} After three weeks of treatment, the animals were anesthetized with sodium thiopental (100 mg kg⁻¹ i.p.) and placed on a heating pad to maintain body temperature at 37° C.¹⁸ After tracheostomy, the left jugular vein and the right carotid artery were cannulated with polyethylene tubing (Portex Ltd., Hythe, England). The carotid cannula (id 0.4, od 0.8 mm) filled with heparinized saline (50 U ml⁻¹) was connected to a pressure transducer to record mean arterial pressure (MAP) using the Power Lab Data Acquisition System (AD Instruments Pvt. Ltd., Sydney, Australia). The left jugular vein cannula (i.d. 0.58, o.d. 0.965 mm) was used to administer acetylcholine (ACh), sodium nitroprusside (SNP), L-NAME, apamin (Apa) and charybdotoxin (ChTx).

Data analysis

Data are displayed as mean and standard error of the mean. Statistical analysis was performed using either

TABLE 1. Effect of saline treatment for 3 weeks on MAP

Group	MAP
<i>Old</i>	
Con (O) (10)	113±2
Sal (O) (10)	116±2
<i>Young</i>	
Con (Y) (12)	114±2
Sal (Y) (12)	133±3 ***
Con (Y) + clof (5)	112±2
Sal (Y) + clof (10)	105±2 †††

MAP: Mean arterial pressure; Con: control; Sal: saline-treated (O): old rats; (Y): young rats.

Numbers in parentheses are numbers of animals

Data are expressed as mean ± SEM

*** *P*<0.001 vs. Con (Y); ††† *P*<0.001 vs. Sal (Y)

Students' t-test or ANOVA, followed by a Newman-Keuls post-hoc test if appropriate. *P*< 0.05 was deemed significant.

Results

Effect of salt and clofibrate on MAP

Saline treatment did not affect MAP in O (116±2 vs. 113±2 mmHg; *n*=10 each) (table 1). In contrast, saline treated Y developed hypertension (133±3 vs. 114±2 mmHg; *n*=12 each, *P*<0.001) which was prevented by co-administration of clofibrate (105±2 mmHg; *n*=10, *P*<0.001 vs. saline). Clofibrate did not affect MAP in control Y (112±2 mmHg; *n*=5).

Effect of salt and clofibrate on PNO_x

Saline treatment did not affect PNO_x in O (2.95 ± 0.17 vs. 3.51 ± 0.53 µM; *n*=4) (figure 1). In contrast, saline treatment induced a significant decrease in PNO_x in Y (2.09 ± 0.26 vs. 4.81 ± 0.36 µM; *n*=6, *P*<0.001). Clofibrate did not affect PNO_x in either the control or the saline treated Y (4.40 ± 0.47, 1.83 ± 0.27 µM; *n*=6).

Effect of salt and clofibrate on aortic superoxide anion production

Saline treatment did not affect aortic superoxide anion production in O (1247.5 ± 265 vs. 1107.5 ± 160

FIGURE 1. Effect of saline and clofibrate treatment on plasma total nitrites + nitrates (PNO_x).
Con and Sal : Control and saline-treated young (Y) and old (O) rats ; clof : clofibrate-treated rats
*** $P < 0.001$ vs. Con (Y).

FIGURE 2. Effect of saline and clofibrate treatment on aortic superoxide anion production.
Abbreviations same as Fig. 1.
*** $P < 0.001$ vs. Con (Y); ††† $P < 0.001$ vs. Sal (Y)

milliunits/mg/min; $n=4$) (figure. 2). In contrast, in Y, saline treatment induced an increase in aortic superoxide anion production (2388.8 ± 40 vs. 1617.3 ± 83 milliunits/mg/min; $n=6$, $P < 0.001$) that was prevented by chronic clofibrate administration (1378.8 ± 64 milliunits/mg/min; $n=6$, $P < 0.001$). Clofibrate treatment did not affect superoxide production in the control Y (1505 ± 65 milliunits/mg/min; $n=6$).

Endothelial dysfunction in saline treated young rats and the effect of L-NAME

ACh induced a concentration dependent decrease in MAP in control O that was similar in both groups of O. L-NAME inhibited ACh evoked fall in MAP response in both the control and saline treated O.

In contrast to O, Y saline treated rats showed decreased ACh evoked responses when compared to the control Y (figure 4a). Furthermore, L-NAME inhibited ACh evoked responses only in control Y. However, SNP induced fall in MAP was similar in both groups of Y (figure 4b). Indomethacin had no effect on responses to ACh or SNP in any group (data not shown).

Effect of Apa + ChTx on ACh and SNP induced fall in MAP responses

Apa+ChTx inhibited ACh induced responses in both the control and saline treated groups of young and old rats (figures 3b and 4b). However, Apa+ChTx did not affect SNP evoked responses in either O or Y.

Clofibrate prevents endothelial dysfunction in saline treated young rats

In Y treated with saline+clofibrate for three weeks, ACh evoked responses were not only normalized but even greater compared to control rats (figure 5). ACh evoked responses were not different in vehicle+clofibrate treated rats (data not shown). Also, SNP evoked responses were not affected by clofibrate treatment.

Apa+ChTx attenuates ACh evoked fall in MAP in saline+clofibrate treated rats

The ACh induced decrease in MAP in saline+clofibrate treated young rats was attenuated by a combination of Apa+ChTx but not by L-NAME (figure 5).

FIGURE 3.
Endothelial function in saline treated (Sal) and control (Con) older Sprague-Dawley rats:
(A) with and without nitric oxide synthase inhibition
†† $P < 0.01$, ††† $P < 0.001$ vs. Con; ††† $P < 0.001$ vs. Sal.
(B) with and without apamin (Apa) + charybdotoxin (ChTx)
ACh: acetylcholine; SNP: sodium nitroprusside
† $P < 0.05$, ††† $P < 0.001$ vs. Con;
‡ $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ vs. Sal.

Discussion

In the present study we confirmed that saline treated young rats develop hypertension and endothelial dysfunction, while older animals do not. It therefore appears that high salt intake does not cause endothelial dysfunction without increasing blood pressure.

FIGURE 4.
Endothelial function in saline treated and control young Sprague-Dawley rats:
(A) with and without nitric oxide synthase inhibition (L-NAME)
** $P < 0.01$, *** $P < 0.001$ vs. Con;
† $P < 0.05$, ††† $P < 0.001$ vs. Con
(B) with and without apamin (Apa) + charybdotoxin (ChTx).
* $P < 0.05$, *** $P < 0.001$ vs. Con;
†† $P < 0.01$, ††† $P < 0.001$ vs. Con
‡ $P < 0.05$, ††† $P < 0.001$ vs. Sal

Clofibrate prevented both hypertension and endothelial dysfunction in young animals. It accomplished the latter by reducing oxidative stress, but more importantly, by enhancing EDHF activity. The ACh induced fall in MAP in clofibrate+saline treated young rats was attenuated by a combination of Apa+ChTx,

FIGURE 5. Effect of chronic clofibrate on saline induced endothelial dysfunction in young rats.

* $P < 0.05$, ** $P < 0.01$ vs. Con; † $P < 0.001$ vs. Sal;

‡ $P < 0.001$ vs. Sal+clof

but not by L-NAME. Hence, the prevention of endothelial dysfunction by clofibrate was mediated, for the most part, through calcium activated potassium channels. This suggests that clofibrate acts via increasing the action of EDHF.

EDHF is poorly understood, but an accepted characteristic is inhibition of its activity by blockade of K_{Ca} channels.^{10,12} Clofibrate could increase the activity of EDHF by increasing its release, prolonging its half-life or potentiating its action. Since the effect of clofibrate is limited to saline treated rats, which are likely producing more EDHF in response to ACh, we believe that clofibrate potentiates EDHF. Clofibrate and other fibrates are peroxisome proliferator activated receptor- α (PPAR α) agonists and have been shown to induce CYP 2C23²² and increase epoxyeicosatrienoic acid production in the kidney.²³ Furthermore, in vitro studies revealed that incubation of endothelial cells with clofibrate for 72 hours causes membrane hyperpolarization, an effect attributed to EET formation.²⁴ EETs induce vasodilation in the mesenteric vasculature²⁵ and also have been identified as EDHF-like in certain vascular beds.¹² Hence, enhancing EET forma-

tion could cause vasodilation, reduced blood pressure and increased EDHF activity.

In some studies, EDHF has been shown to compensate completely⁸ for reduced nitric oxide activity and maintain normal endothelium dependent responses in aortic rings²⁶ and mesenteric vessels²⁷ of saline treated rats. However, other studies have shown reduced endothelium dependent relaxation responses in spino-trapezius vessels⁵ and aortic rings.⁶ These reported differences could be a result of differences in animal age, type of vascular bed studied, amount and duration of salt intake or other factors.

In our high salt intake young animals, the observed decreases in endothelium dependent responses could be due to a combination of decreased NO production and lack of complete compensation by EDHF. The former is suggested by the reduction in PNO_x and the lack of effect of L-NAME on ACh mediated responses. Apa+ChTx, but not L-NAME further inhibited the already attenuated ACh-induced responses in saline treated young rats, suggesting a role for calcium-activated potassium channels in their residual endothelium dependent relaxation. Therefore, increased EDHF activity partially, but not completely, compensates for the reduced nitric oxide activity.

Indomethacin (10 mg/kg i.v.) an inhibitor of prostaglandin H synthase did not affect the percent fall in MAP in response to ACh in any of the animals. We suggest that prostacyclin does not play a major role in determining responses to ACh in Sprague-Dawley rats.

An important observation in our study is that saline treated young rats had increased aortic superoxide production, a marker of oxidative stress, and a phenomenon observed previously.⁶ Clofibrate prevented this increase. Even though clofibrate reduced superoxide anion production in saline treated rats, it failed to restore to normal the total PNO_x, suggesting that there was decreased nitric oxide production in saline treated rats in addition to quenching of nitric oxide by superoxide. These results are in agreement with a previous study reporting decreased NO_x in the perfusate of iso-

lated kidney from Dahl salt-sensitive rats.²⁸ Salt loading attenuates the conversion of L-arginine to nitric oxide in the renal vascular endothelium of salt-sensitive patients with essential hypertension.²⁹ It has been well documented that the L-arginine – NO pathway is impaired in salt-sensitive experimental hypertension by reduced bioavailability of endothelial NO.^{30,31} The suppressant effect of high salt intake on plasma NO_x concentration could occur by several mechanisms, including altered transport of L-arginine through the endothelial membrane, decreased activity of nitric oxide synthase, or an increased breakdown of nitric oxide.³² 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.³³ The present study does not allow us to distinguish among these possibilities.

A limitation of our study became evident when we found that clofibrate prevented both hypertension and endothelial dysfunction in saline treated young animals. It might be argued that simply lowering the blood pressure (or at least preventing hypertension) might prevent endothelial dysfunction. However, we think that the effects of clofibrate on blood pressure and endothelial function are separate. In our previous study we found that a single dose of clofibrate given to saline treated young rats, restored endothelial function but had no effect on blood pressure.¹⁵ It is also generally recognized that endothelial dysfunction can occur with aging, hyperlipidemia and obesity without a measurable change in blood pressure.³⁴ Finally, some antihypertensive drugs lower blood pressure but do not affect endothelial function.³⁵

The clinical significance of our results is uncertain. Salt sensitivity, often defined as a greater than 10 mmHg increment in blood pressure on switching to a high salt diet, occurs more commonly in older humans.³⁶ This has been attributed to inability to excrete salt due to nephron dropout with ageing. Salt induced endothelial dysfunction appears to occur in humans with or without an increase in blood pressure.³⁷ Therefore, mechanisms for hypertension

and endothelial dysfunction may be species dependent. It is known that 20-HETE can be produced by human tissues. The human homologues of rat CYP4A are CYP4A11 and CYP4F2.³⁸ Whether expression of one or both of these is increased by fibrates awaits further investigation.

In summary, we have shown that endothelial function is reduced in young S-D rats given saline, likely through reduced nitric oxide availability. Their residual endothelial function is heavily dependent on EDHF. Clofibrate restores endothelial function partly by reducing oxidative stress and nitric oxide quenching, but in the main through enhancing EDHF action.

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