Effects of salsalate therapy on recovery from vascular injury in female Zucker fatty rats

Running Title: Salsalate therapy for recovery from vascular injury

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**Objective:** Salsalate is a dimeric form of salicylic acid that has been shown to have anti-inflammatory activity, reduce glucose levels, insulin resistance and cytokine expression. However the effect of salsalate on vascular injury has not been determined.

**Research Design and Methods:** To investigate the effect of salsalate on vascular injury and repair in a rat model of carotid artery balloon catheter injury. Salsalate treatment was started in female Zucker fatty rats (insulin resistant) one week before carotid artery balloon catheter injury and continued for 21 days at which time the animals were sacrificed and studied.

**Results:** Treatment with salsalate significantly decreased the I/M ratio and upregulated the expression of aortic eNOS, p-eNOS (ser 1177), MnSOD and reduced serum IL-6 with concomitant down regulation of NFκB subunit p65, and VEGF expression in the balloon injured carotid artery of female Zucker fatty rats.

**Conclusions:** The present study shows that salsalate treatment decreases vascular damage caused by balloon catheter injury in female Zucker fatty rats. The beneficial effect of salsalate on vascular injury was associated with upregulation of eNOS, p-eNOS, and MnSOD which reduce oxidative stress and have anti-inflammatory properties as evidenced by reduction in serum IL-6 and the down regulation of VEGF and NFκB which promote inflammation without changing glucose levels. These results suggest that salsalate may be useful in reducing vascular injury and restenosis following interventional revascularization procedures.

Type 2 diabetes (T2D) mellitus is an important, independent risk factor for heart disease (1), and it is reported that cardiovascular disease (CVD) accounts for up to 80% of excess mortality in patients with T2D. Further, these patients have a 2-fold increased risk of death independent of other known cardiovascular risk factors (2,3). More than 17 million Americans suffer from either type 1 or T2D and newly diagnosed cases are increasing. In addition, from an epidemiologic view point the pre-diabetic population is considered to be the most important group, comprising several thousand individuals (4). Insulin resistance (IR) plays a key role in the pathogenesis of T2D, and is a hallmark of metabolic disorders including dyslipidemias, obesity, hypertension, and CVD (5-7). In patients with diabetes interventional vascular procedures such as balloon angioplasty and stent placement have been shown to result in greater restenosis rates (8). These procedures cause mechanical injury to the artery, leading to a cascade of cellular and inflammatory events. Intimal hyperplasia (IH) is an underlying cause of restenosis and vessel narrowing; however the mechanisms causing IH are not well understood (9).

Obesity, diabetes and CVD have been shown to be associated with chronic low-level vascular inflammation (10). The potential for suppression of inflammation in the treatment of these conditions is an active area of investigation.

Salicylates are among the most commonly used nonsteroidal anti-inflammatory drugs (11). The reduction of glycosuria by high doses of sodium salicylate (5.0–7.5 g/d) was shown in diabetic patients having “the milder form of the disease,” presumably T2D (12-14). In recent studies, the hypoglycemic actions of salicylates were investigated, and the molecular target was shown to be the IkB kinase complex β (IkKB)/nuclear factor κB (NF-κB) pathway (15,16). This pathway is a critical regulator of proinflammatory signals (10).
Salsalate belongs to the drug class of salicylates, and is the dimeric form of salicylic acid (17). This compound is known to inhibit the formation of pro-inflammatory cytokines including TNF-α, IL-6 and IL-7 (18). Salsalate is known to inhibit the expression of vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1), and endothelin-1, which are produced by the endothelium in response to injury.

Recent studies have shown that increased expression of vascular endothelial growth factor (VEGF; a powerful mitogen for endothelial and vascular smooth muscle cells) may lead to increased neointimal hyperplasia after angioplasty (19,20). Moreover, the use of an anti-VEGF (bevacizumab) coated stent decreased IH post angioplasty in rabbits (21). Novotny and coworkers have reported that the expression and secretion of VEGF is mediated by NF-κB (22). Based on the known inhibitory effect of salsalate on NF-κB, it is possible that this agent may decrease the expression of VEGF leading to decreased IH after angioplasty, however, this hypothesis has not yet been tested. In addition, insulin stimulates VEGF expression and animal models that are hyperinsulinemic have increased IH associated with increased VEGF expression after angioplasty (19). Salsalate may decrease inflammation by inhibiting of NF-κB and decrease IR which in turn may result in a decrease in expression of VEGF.

In view of the beneficial effects of salsalate on glucose metabolism, the NF-κB/IκKβ pathway, and inflammation, we investigated the effect of salsalate on the inflammatory response that follows vascular injury. Additionally, as inflammatory responses have been linked to the production of reactive oxygen species [ROS] (23), we ascertained if salsalate treatment had antioxidant effects that had a role in the vascular recovery process. Finally, we explored the potential effects of salsalate on VEGF expression, an important growth factor. In the present communication we report the effect of salsalate on the balloon catheter injured carotid artery in an animal model of IR.

**MATERIALS & METHODS**

The protocol used for animal experiments in the present study was approved by the Institutional Animal Care and Use Committee of the Tulane University School of Medicine, New Orleans, LA. Nine to ten week old female Zucker-fatty rats were obtained from Harlan Sprague Dawley Inc., Indianapolis, IN. The Zucker-fatty rats are obese with normal glucose but they exhibit hyperphagia, hypertriglyceridemia, hyperinsulinemia, and reduced glucagon levels (24). This model will therefore facilitate in avoiding the confounding effects of major changes in blood glucose concentration. In addition, insulin resistant models are associated with a propensity for neointimal proliferation, an observation not seen in insulin deficient models with or without exogenous insulin administration (9, 25).

The rats were housed individually with free access to food and water, and were maintained in a temperature and humidity regulated room with a 12-hour light/dark cycle. After an acclimatization period, the rats were randomly divided into control and salsalate-treatment group. The control group received food at libitum, and the animals in the experimental/salsalate group received 300 mg/d salsalate mixed in the food given ad libitum.

Treatment with salsalate was initiated one week prior to balloon catheter injury of the left carotid artery, and continued for an additional 21 days after injury. The body weight and blood glucose levels (fasting) were determined at weekly intervals. Blood glucose levels were measured with a glucometer using tail vein blood samples, and typically the blood sample was taken after overnight fasting of the rats.
**Balloon Catheter-Induced Injury:** After one week of feeding the salsalate containing diet, balloon injury was induced as previously described (25,26). Briefly, the rats were anesthetized with 5% isoflurane in oxygen, and the femoral artery was approached. Using standard methods, a Maverick over the wire catheter (20 X 2.0 mm; Boston Scientific, Natick, MA) was passed up the aorta to the carotid artery under fluoroscopic control. The balloon was inflated to 4.0 atmospheres and held for exactly 20 seconds before partial deflation. The pressure was then reduced to 2.0 atmospheres and the balloon was dragged to the descending aorta for denudation of the endothelium. At this point, the catheter was completely deflated and the pressure made negative. The catheter was then gently withdrawn while simultaneously tying the artery with sterile 7/0 silk suture. The incision was closed with Vicryl sutures and the animals were allowed to recover before being returned to the vivarium. The rats were maintained on their respective diet (control or salsalate added) for 3 weeks after the procedure and sacrificed on day 22 after the procedure. The animals were euthanized in a CO\textsubscript{2} chamber, and a blood sample was taken in addition to harvesting injured and the contralateral carotid arteries (for comparison), aorta, heart and other tissues. The blood and tissue samples were processed for biochemical and histological analysis. Both injured and uninjured carotid arteries were processed for sectioning. The carotid artery sections were H&E stained and the intima/media ratio (I/M) was measured at a magnification of X10. Computerized digital microscopic software (Image-Pro plus 4) was used to obtain measurements of the intimal and medial areas by a previously described method (25,26). The I/M ratio was calculated for all specimens for comparison between treatment groups.

**Biochemical parameters:** At the time of sacrifice, a blood sample, both the injured and the contralateral carotid arteries, the aortic arch and other tissue samples were harvested. The blood and tissue samples were processed by standard methods and appropriately stored for subsequent biochemical analysis. The serum was used for the analysis of interleukin-6 (IL-6). Protein was isolated from aorta for Western blot analysis of endothelial nitric oxide synthase (eNOS), phosphorylated-eNOS, (ser-1177), NF-κB subunit p65 and Manganese superoxide dismutase (MnSOD or SOD2). Following endothelial denudation, eNOS and p-eNOS, important regulators of vascular homeostasis, were evaluated for alterations in endothelial function and recovery; SOD2 was analyzed to ascertain if salsalate treatment was modulating antioxidative defense mechanism. Balloon catheter induced endothelial denudation injures the vessel evoking an inflammatory response. Therefore in evaluating the inflammatory status in serum from salsalate treated animals, IL-6 and aortic NF-κB subunit p65, a well established biomarker were assayed (27). Serum IL-6 was analyzed by enzyme immuno assay method using Quantikine® IL-6 immunoassay kit obtained from R & D systems (Minneapolis, MN).
**Immunohistochemistry for VEGF:** The immunohistochemical analysis for VEGF was carried out using previously described methods (19). Cross sections of formalin-fixed, paraffin-embedded carotid arteries were used for analysis. The carotid artery sections were obtained as described in the morphometric analysis section. Each sample was deparaffinized in xylene and gradually rehydrated using decreasing percentages of ethanol. After blocking endogenous peroxidase with 30% H$_2$O$_2$ in methanol for 20 minutes, an antigen retrieval step was added by microwaving the samples in citrate buffer (pH 6.0) for 30 minutes. The samples were then washed 3 times with phosphate buffered saline (PBS), followed by incubation with Chem Mate blocking antibody solution (DAKO, Carpentaria, CA) for 30 minutes. The samples were then incubated for one hour at room temperature with murine anti-VEGF (Oncogene, San Diego, CA), diluted 1:50 in PBS. The samples were then washed and incubated with ready to use DAKO EnVision+, Peroxidase, for 30 minutes at room temperature. After incubation, the slides were rinsed in PBS, exposed to stable diaminobenzidine tetrahydrochloride (DAB) and the reaction was monitored using light microscopy. The sections were then counterstained with Mayer’s hematoxylin.

For quantification, 4 sections from each carotid artery were analyzed. The images were recorded using a SPOT cooled color digital camera linked to a computer. Digitized software (AN@lysis) was used to measure the staining intensity of the neointima. The total intensity of staining of all the pixels in the neointimal area were measured and then divided by the total number of pixels in the neointima to obtain a mean staining density value.

**Western Blot Analysis:** Aortic tissue was homogenized and incubated in a lysis buffer. The BCA protein assay (Pierce, Rockford, IL) was used to quantify protein (28). Proteins of equal quantities were electrophoresed on 4-20% gradient gels (Jule Inc, Milford CT, USA). The protein was transferred to a nitrocellulose membrane (GE Healthcare, Piscataway, NJ). The membrane was blocked for 1 hour in 5% milk in tris buffered saline (TBS) + 0.1% Tween (TBST). The membrane was then washed three times for 5 minutes and incubated with primary antibodies which were purified mouse anti-eNOS/NOS type III (BD Transduction Labs), NF-κB-p65 and MnSOD at a dilution of 1:1000 overnight. The membrane was washed three times with TBST, and bound antibody was detected using anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology) and anti-rabbit IgG-HRP (Santa Cruz), respectively. Lumiglo chemiluminescense (KPL) was used to detect bound protein, and the protein was quantified using QuantityOne® analysis software (Bio-Rad). These standard methods have been routinely used by other investigators (29,30). For the Western blot analysis of p-eNOS (ser 1177), goat polyclonal IgG obtained from Santa Cruz Biotechnology, (Santa Cruz, CA) was diluted 1:1000 for incubation overnight. The secondary antibody was donkey anti-goat IgG-HRP obtained from Santa Cruz Biotechnology, (Santa Cruz, CA), and was used at a dilution of 1:2000. For analysis of MnSOD, the tissue was homogenized in lysis buffer and centrifuged at 10,000 x g for 10 minutes at 4°C. Protein concentration of these homogenates was determined using the BCA method (Pierce, Rockford, IL, USA). Equal quantities of protein were electrophoresed on 4-20% gradient gels (Jule Inc, Milford CT, USA). The protein was then transferred to a nitrocellulose membrane (GE healthcare). The membrane was blocked for 1 hour in 5% milk in tris buffered saline (TBS) + 0.1% Tween (TBST). The membrane was washed three times for 5 minutes each and then incubated in polyclonal MnSOD antibody (Cayman...
Salsalate therapy for recovery from vascular injury

Chemical, Ann Arbor, MI) at a dilution of 1:1000 overnight. The membrane was washed with TBS and then bound antibody was detected using anti-rabbit IgG-HRP secondary antibody (Santa Cruz biotechnology, Santa Cruz, CA) at a dilution of 1:2000 for 30 minutes. For the detection and quantitation of MnSOD, Lumiglo chemiluminescent detection system (KPL, Gaithersburg, MD) was used. Densitometric analysis of the autoradiographs was carried out with QuantityOne analysis software (Bio-Rad, Hercules, CA).

Statistical Analysis: Statistical analysis was performed using the Sigma Stat (SSPS, Chicago, IL) and GraphPad software programs (GraphPad Software, La Jolla, CA). One way ANOVA, unpaired t test, and all pair wise multiple comparison procedures (Tukey test) were used. Data are reported as mean +/- SEM. Statistical significance was set at an α of <0.05.

RESULTS
The data on body weight gain, morphometric and biochemical analysis of control and salsalate treated Zucker fatty rats are presented in table 1. The gain in body weight over the experimental period and fasting glucose levels were not significantly different, however, the I/M ratio, serum IL-6 and the expression of eNOS, p-eNOS, MnSOD and NFκB subunit p65 were significantly different.

Intima/Media Ratio (I/M): The morphometric measurement of the carotid artery cross-sections from the balloon catheter-injured and untreated or control and salsalate-treated groups of rats had an I/M ratio of 0.9 ± 0.2 vs 0.19 ± 0.11 (P<0.05). Representative sections of H&E stained carotid arteries from uninjured (A), injured and untreated/control (B) rats are presented in figure 1. The treatment with salsalate resulted in significant attenuation of the hyperplastic response. A representative H&E stained carotid artery section from balloon catheter injured and salsalate treated rats is shown in figure 1C. The quantitation of IH is shown in figure 1D. These results suggest that the attenuating effect on IH could be attributed, at least in part, to treatment with salsalate.

VEGF Expression: VEGF intimal staining intensity was measured in carotid sections as described in the methods section. Intimal VEGF protein staining intensity was significantly decreased by 22% in salsalate treated rats (62+/-7) vs untreated rats (78+/-6). Representative sections of immunostained carotid artery sections from balloon catheter injured, untreated/control (A), and balloon catheter injured and treated with salsalate (B) are shown in figure 2.

Western Blot Analysis –eNOS, p-eNOS, and NFκB-p65:
The relative density units (RDU) obtained from the densitometric scan of the Western blots (after normalizing expression levels to corresponding β-actin) indicate a significant increase in the expression of eNOS in aortic arch protein samples from salsalate-treated rats (0.15 ± 0.07) as compared to the control group (0.03 ± 0.01); p<0.05, indicating a 5 fold increase (0.15/0.03) (Fig 3). The p-eNOS expression was increased in aortic arch samples from salsalate treated rats (2.0 ± 0.5) compared to expression in control rats (0.2 ± 0.06); p<0.005, indicating a 10 fold increase (2.0/0.2). The ratio of p-eNOS to eNOS in the control animals was 6:1 (0.2/0.03) and 13:1 (2.0/0.15) in the rats treated with salsalate (Fig 4). These results indicate that there was a significant increase in the level of phosphorylated eNOS in salsalate treated rats.

The results of Western blot analysis of the aortic arch for NF-κB sub unit p65 showed a significant decrease in expression in the salsalate-treated group (mean relative densities of the salsalate-treated and control groups: 0.159 ± 0.054 versus 0.600 ± 0.110; p<0.05). This decrease indicates that down regulation of the NF-κB-p65 pathway may
have a role in the beneficial effect of salsalate in attenuating the inflammatory response following carotid artery balloon catheter injury (Fig 5).

**Serum IL-6:** The serum IL-6 levels were assayed by enzyme immune assay methods (per manufacturer’s instructions). The serum IL-6 levels were significantly decreased in rats treated with salsalate vs controls (88 ± 1.8 vs 99.2 ± 2.9) and suggest that salsalate produced a better recovery from balloon injury by inducing an anti-inflammatory effect.

**Western Blot Analysis – MnSOD:** MnSOD expression was analyzed in the aortic arch by Western blot analysis, and there was a clear increase in the expression of this antioxidant defense enzyme in salsalate treated rats (mean relative densities of the salsalate was 1.2 ± 0.2 and control was 0.67 ± 0.07; p<0.05), suggesting that salsalate therapy could reduce the expression of reactive oxygen species and tissue injury. The Western blot analysis of MnSOD is shown in figure 6.

**DISCUSSION**

In the present study we observed that Zucker fatty rats pretreated with salsalate and subjected to carotid artery injury and placed on the treatment regimen, exhibited a marked reduction in the hyperplastic vascular response normally seen in untreated animals. Compared to the control group, the salsalate-treated rats had I/M ratios of 0.19 ± 0.11 versus 0.9 ± 0.2, p<0.05. These rats are obese with normal glucose levels but exhibit hyperphagia, hypertriglyceridermia, hyperinsulinemia, and reduced glucagon levels (24). This model therefore allowed us to avoid the effects of major changes in blood glucose concentration. In order to study the effects of an intervention on vascular injury, this model is advantageous as it has a propensity for neointimal proliferation that is not seen in insulin deficient rats and is independent of insulin administration (9,25).

Intimal thickening in response to injury is associated with inflammation and proliferation of vascular smooth muscle cells (VSMCs) and has been observed in diabetes and with insulin resistance (6). Studies have shown that vascular injury in response to balloon angioplasty and stent placement, results in greater restenosis rates in patients with diabetes mellitus (8,9).

Ongoing clinical trials evaluating the effects of salsalate treatment and the use of salsalate in the Type 2 Diabetes (TINSAL-T2D) trial will provide further information on the effect of salsalate (TINSAL-T2D 2009) and the metabolic syndrome. Our results providing information on the mechanism of the beneficial action of salsalate on vascular injury may be relevant to T2DM population.

The mechanisms mediating intimal thickening that occur in response to inflammation are less well understood than those originating from vascular injury. Although controversial, a role for nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway has been suggested. NF-κ light chain enhancer is known to induce the synthesis of pro-inflammatory cytokines and chemokines (TNF-α, IL-6, IL-1β, etc.) and to stimulate the recruitment of macrophages to adipose tissue (27). The upregulation of pro-inflammatory cytokines and chemokines [TNF-α, IL-6, IL-1β, etc.] by NF-κB during inflammation has been documented (31). The effect of lowering circulating IL-6 by salsalate as observed in our studies provides support for the role of NF-κ light chain enhancer as well.

Although the mechanism of action of salsalate is not well understood, it is possible that this agent suppresses NF-κB via inhibition of IKKβ. Salsalate has been used for decades to treat inflammation and has a good safety profile, including a lower bleeding risk when compared to aspirin (17). It was reported that after administration of 3 grams of salsalate for seven days, the fasting plasma glucose
concentration was significantly decreased when assessed by the oral glucose tolerance test (OGTT). However, Koska and coworkers did not find a significant change in plasma insulin concentration (17). Another study, examined the efficacy of salsalate in reducing glycemia and insulin resistance while attempting to define the mechanism of action of salsalate, and validating NF-κB as a primary target in the treatment of diabetes (32). Further, it was reported that during the intravenous glucose tolerance test there were increases in insulin and C-peptide concentrations in patients given 4.5 gms salsalate. These observations support the hypothesis that higher doses of salicylates effectively reduce insulin clearance (16,32).

Goldfine et al., demonstrated that salsalate effectively inhibits NF-κB activity, a well-recognized marker of inflammation (32). These effects are thought to be mediated by inhibition of IKKβ, an upstream kinase necessary for the activation of NF-κB (15,16).

After observing the beneficial effects of salsalate on the injured vessel segment, we investigated the effect of this agent on vascular biology in the post-injury/recovery phase. Western blot analysis for p-eNOS and eNOS expression in vascular tissue showed an upregulation. The expression of eNOS in the salsalate treated rats was increased 5 fold compared to control rats. The p-eNOS in rats treated with salsalate was increased 10 fold compared to the control animals showing a higher level of phosphorylated eNOS in the salsalate treated rats. The ratio of p-eNOS to eNOS in the control animals was 6:1 (0.2/0.03) and 13:1 (2.0/0.15) in the rats treated with salsalate. The upregulation of eNOS and p-eNOS would suggest that salsalate may have a favorable effect on endothelial function due to increased formation of NO and is in agreement with previous studies (33).

In addition to changes in eNOS expression, a response to balloon catheter injury that is often seen is an inflammatory response (27). To evaluate this component, we assessed circulating levels of IL-6 in blood samples collected at the end of the experiment. IL-6 levels were significantly lowered in rats treated with salsalate when compared to levels in control rats providing evidence in support of the anti-inflammatory role of salsalate. Further, Western blot analysis for the expression of the NF-κB subunit p65 was performed. The association between NF-κB and inflammation is well known as it has been shown to induce the upregulation of pro-inflammatory cytokines and chemokines [TNF-α, IL-6, IL-1β, etc.] (31). Western blot analysis for p65 in the aortic arch of the injured, salsalate-treated rats when compared to control rats showed a significant decrease in expression. It is therefore possible that treatment with salsalate suppressed the activity of NF-κB, suggesting a mechanism for the attenuation of the inflammatory response.

This study shows that VEGF expression was decreased in the intima after treatment with salsalate, which corresponds to a reduction in intimal hyperplasia. The suppression of VEGF by insulin has been shown in humans (34), and this has been substantiated by studies in type 1 diabetics (35). The role of VEGF and its action may be related to the pathophysiological condition.

The exercise training associated reduction of IL-6 levels in Zucker diabetic fatty rats has been reported by Teixeira de Lemos and coworkers (36). In the present study we also observed a reduction of serum IL-6 in Zucker fatty rats subjected to balloon catheter injury and treated with salsalate, and may be related to a reduction in NF-κB expression and vascular inflammation. Oxidative stress and the generation of ROS and their role in tissue repair are well documented. The trauma of balloon injury and related inflammatory response is a stimulus for the generation of ROS. The redox status of the cell is
Salsalate therapy for recovery from vascular injury

maintained by antioxidant enzymes such as superoxide dismutase, catalase, glutathione S-transferase and other substances such as glutathione, vitamins E, C and A, which help in the suppression of ROS. MnSOD is a critical antioxidant enzyme that protects against superoxide anion generated by normal cellular respiration, and an inflammatory response was evaluated and found to be upregulated and may be involved in the beneficial effect of salsalate (37). It is our hypothesis that upregulation of MnSOD should decrease ROS levels. A limitation of our study is the absence of the measurement of ROS.

Salsalate has been shown to improve glucose and lipid homeostasis and has been used for targeting inflammation in the treatment of insulin resistance and T2D (32). Our data as evidenced by a reduction in NFκB-p65, increased expression of MnSOD and a significant reduction of IH (typically seen after vascular injury in insulin resistant animal models) suggest that salsalate has both anti-inflammatory as well as antioxidative properties. It is relevant to note that insulin, the best known antidiabetic drug, has been reported to have a protective effect (after vessel injury) on smooth muscle cells and vascular endothelium resulting in the inhibition of neointimal growth (38).

In summary, the data obtained in our study suggest that salsalate is capable of reducing intimal hyperplasia following balloon catheter-induced injury. It is suggested that this effect of salsalate is due, at least in part, to its favorable effect on endothelial eNOS/p-eNOS expression and upregulation of antinflammatory and antioxidant activity. The results with salsalate on IH are important and may improve our understanding of vascular complications associated with hyperglycemia and interventional vascular procedures.

Author Contributions: SNM contributed to the discussion and wrote manuscript. CVD researched data and contributed to discussion. NWB researched data. R-C S.H, DBC, AMB, JSD, JM, and DBM researched data. PJK and VAF contributed to discussion and reviewed/edited manuscript.

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Salsalate therapy for recovery from vascular injury

Salsalate therapy for recovery from vascular injury


| Table 1. Biochemical and morphometric analysis of carotid artery injured female Zucker fatty rats treated with salsalate |
|---------------------------------------------------------------|-------------|------------------|
| Gain in body weight over the experimental period              | Control     | Salsalate treated | p value |
| Fasting blood glucose (mg/dL)                                 | 89 ± 8      | 90.7 ± 15.5      | NS      |
| I/M ratio                                                    | 0.9 ± 0.2   | 0.19 ± 0.11*     | <0.05   |
| IL-6 (pg/ml)                                                 | 99.2 ± 2.9  | 88 ± 1.8*        | <0.05   |
| NFkB-p65 [Relative density units; RDU]                        | 0.6 ± 0.1   | 0.16 ± 0.05*     | <0.05   |
| MnSOD [ RDU]                                                 | 0.67 ± 0.07 | 1.2 ± 0.2*       | <0.05   |
| eNOS [ RDU]                                                  | 0.03 ± 0.001| 0.14 ± 0.07*     | <0.05   |
| p-eNOS (ser 1177) [ RDU]                                     | 0.2 ± 0.06  | 2.0 ± 0.5†       | <0.005  |
Figure legends:

**Figure 1.** Representative sections of H&E stained carotid arteries from uninjured (A) and injured and untreated/control (B) Zucker fatty rats. The hyperplastic response that follows injury was significantly reduced when the rats were treated with salsalate (C), quantitation of the reduction in intimal hyperplasia is depicted in D. Balloon catheter injury results in a hyperplastic response in the injured segment of the artery and salsalate produces a significant attenuation of the response. The reduction in intimal hyperplasia could be attributed, at least in part, to treatment with salsalate.

**Figure 2.** VEGF protein immunostained carotid artery sections of injured, untreated/control (A), and injured and treated with salsalate (B) Zucker fatty rats. The intimal staining intensity was significantly decreased by 22% in salsalate treated rats (62+/-7) vs untreated rats (78+/-6); p<0.05.

**Figure 3.** The RDU obtained from the densitometric scan of the Western blots of eNOS (after normalizing expression levels to corresponding β-actin) showed a marked increase in eNOS expression in aorta samples from salsalate-treated rats when compared to the control group. The mean relative density of scans from control rats that were balloon catheter injured and not treated was 0.027 ± 0.009; the mean relative density of the salsalate-treated rats was 0.148 ± 0.066, and was significantly different- p<0.05. Compared to the control rats the expression of eNOS in the salsalate treated rats was increased 5 fold (0.15/0.03).

**Figure 4.** The RDU obtained from the densitometric scan of the Western blots for the expression of phosphorylated-eNOS (ser 1177) (after normalizing expression levels to corresponding β-actin) in aortic tissue showed an upregulation of p-eNOS by salsalate therapy. There was a significant difference in the mean RDU between the control and salsalate treated rats (0.2 ± 0.06 versus 2.0 ± 0.5); p<0.005. Compared to the control rats the expression of p-eNOS was increased 10 fold (2.0/0.2). The ratio of the expression of p-eNOS to eNOS in the control animals was 6:1 (0.2/0.03) and in the salsalate treated rats was 13:1 (2.0/0.15).

**Figure 5.** The Western blot analysis of aortic arch NF-κB sub unit p65 indicated a significant decrease expression in the salsalate-treated group (mean relative densities of the salsalate-treated and control groups: 0.1599 ± 0.1092 versus 0.6003 ± 0.11; p=0.01). The data is indicates that salsalate treatment reduced the inflammatory response following the balloon catheter injury of the carotid artery.

**Figure 6.** The MnSOD expression of aortic arch protein in balloon catheter injured and untreated/control Zucker fatty rats and balloon catheter injured and treated with salsalate. There was a significant increase in the mean relative density in salsalate treated rats when compared to control rats (control was 0.673 ± 0.07 and salsalate 1.2 ± 0.2); p<0.05. The increase in the expression of this antioxidant defense enzyme suggests that salsalate therapy enhances reactive oxygen species quenching thereby reducing tissue injury.
Salsalate therapy for recovery from vascular injury

Figure 1

A

B

C

D

Effect of salsalate on I/M ratio in catheter injured carotid arteries of Zucker fatty rats

Figure 2

Balloon injured and untreated vessels

Balloon injured and salsalate treated vessels

A

VEGF staining intensity 78 ± 6

B

VEGF staining intensity 62 ± 7

p<0.05

Figure 3
Figure 4

Expression of eNOS and p-eNOS (ser-1177) in aorta of carotid injured control and salsalate treated Zucker fatty rats

![Graph showing relative density units for eNOS and p-eNOS expression in control and salsalate treated rats.](image)

Control | Salsalate
---|---

* p<0.05
Figure 5

Expression of NFkB-p65 in aorta of carotid injured control and salsalate treated Zucker fatty rats

![Bar chart showing expression levels of NFkB-p65](image)

Relative density units

- Control
- Salsalate

* p<0.05

Figure 6

Expression of MnSOD in aorta of carotid injured control and salsalate treated Zucker fatty rats

![Bar chart showing expression levels of MnSOD](image)

Relative density units

- Control
- Salsalate

* p<0.05