Cyclooxygenase-2 inhibition restored endothelium-mediated relaxation in old obese Zucker rat mesenteric arteries

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Metabolic syndrome is associated with reduced endothelial vasodilator function. It is also associated with the induction of cyclooxygenase-2 (COX2), which produces vasoactive prostanoids. The frequency of metabolic syndrome increases with age and aging per se is a risk factor associated with reduced endothelium-mediated relaxation. Nevertheless, the combined effect of aging and metabolic syndrome on the endothelium is less known. We hypothesized that COX2 derived prostanoids may affect endothelium function in metabolic syndrome associated with aging. We used obese Zucker rats, a model of metabolic syndrome. First order mesenteric arteries were isolated from 4- and 12-month-old rats and acetylcholine (endothelium)-dependent relaxation determined using wire-myography. Endothelium-mediated relaxation, impaired in young Zucker rats (89 versus 77% maximal relaxation; lean versus Zucker), was further reduced in old Zucker rats (72 versus 51%, lean versus Zucker). The effect of the nitric oxide-synthesis inhibitor L-NAME on the relaxation was reduced in both young and old Zucker rats without change in eNOS expression level. COX inhibition (indomethacin) improved acetylcholine-mediated relaxation in old obese rats only, suggesting involvement of vasoconstrictor prostanoids. In addition, COX2 inhibition (NS398) and TxA2/PGH2 receptor blockade (SQ29548) both improved relaxation in old Zucker rat arteries. Old Zucker rats had the highest TxB2 (TxA2 metabolite) blood level associated with increased COX2 immunostaining. Chronic COX2 blockade (Celecoxib, 3 weeks) restored endothelium-dependent relaxation in old Zucker rats to the level observed in old lean rats. Thus the combination of aging and metabolic syndrome further impairs endothelium-dependent relaxation by inducing an excessive production of COX2-derived vasoconstrictor(s); possibly TxA2.

Keywords: resistance arteries, metabolic syndrome, cyclooxygenase-2, aging, vasodilatation, endothelium

INTRODUCTION

Obesity is a fast growing problem worldwide (James, 2004) and is associated with an increasing risk of cardiovascular morbidity and mortality, especially when it is associated with other risk factors in the metabolic syndrome (Hu et al., 2004). However, the root causes of cardiovascular dysfunction associated with the metabolic syndrome still remain poorly defined (Krentz et al., 2009). The endothelium regulates vascular tone through the production of nitric oxide (NO), prostacyclin and hyperpolarizing factors. The endothelium is affected in metabolic syndrome (Eckel et al., 2005; Frisbee and Delp, 2006). Indeed, studies in human (Arkin et al., 2008) or in obese Zucker rats, a model of hyperphagia-induced metabolic syndrome, have reported endothelial dysfunction in several vascular beds (Zanchi et al., 1995) associated with a reduced NO bioavailability (Bohlen, 2004). Resistance arteries are strongly affected in metabolic disorders (Frisbee and Stepp, 2001; Stepp et al., 2004; Frisbee and Delp, 2006; Krentz et al., 2009) due to increased reactive oxygen species (ROS) production and consequently reduced NO bioavailability (Phillips et al., 2005; Busija et al., 2006; Roberts et al., 2006). Several studies suggest that cyclooxygenase (COX) derivatives might also be involved in this vascular impairment. Indeed, metabolic syndrome is also associated with increased thromboxane A2 (TXA2)-mediated vasoconstriction and endothelium dysfunction (Xiang et al., 2006, 2008). These studies suggest that COX2 might be involved in this production of TXA2.

Aging, per se is also associated to a gradual endothelium dysfunction both in human (Egashira et al., 1993; Taddei et al., 1995) and animal models of aging (Koga et al., 1989; Kung and Luscher, 1995). Again, an excessive ROS production reduces NO bioavailability (Wenzel et al., 2008; Herrera et al., 2010). Nevertheless, the involvement of COX2-derived prostanoids remains a matter of controversy. Several studies have reported an increased COX2-derived TXA2 production with aging, attenuating endothelium-mediated vasorelaxation (Koga et al., 1989) while other studies (Kung and Luscher, 1995) argued for an increase ROS production only. A recent study using a mouse model of obesity reported that aging up-regulates expression of inflammatory mediators in adipose tissues and showed an increased COX2 expression level (Wu et al., 2007). Nevertheless, no study has yet investigated the concomitant effect of aging and metabolic syndrome on the endothelium and its vasodilator capacity. The purpose of the present study was thus
to assess the effect of aging associated with metabolic syndrome on endothelium-mediated relaxation. We hypothesized that in old obese rats COX2 derived prostanoids may further impair endothelium-mediated dilation. We compared acetylcholine-mediated relaxation in first order mesenteric arteries isolated from young and old obese Zucker rats, a model of metabolic syndrome, and defined the mechanism involved in the alteration observed.

**MATERIALS AND METHODS**

**ANIMALS**

Three- and 12-month-old male lean and obese Zucker rats were purchased from Charles River (L’Arbresles, France) and anesthetized (Isoflurane, 2.5%). The right femoral artery was then cannulated for blood pressure measurement as previously described (Ben Driss et al., 2000). Animals were then sacrificed by CO2 inhalation after collecting blood on heparin. The gut excised and the mesenteric arteries gently dissected. Blood glucose was measured as previously described (Frisbee and Stepp, 2001; Katakam et al., 2005; Bouvet et al., 2007).

In a separated series of experiments 12-month-old male lean and obese Zucker rats were treated with the COX2 inhibitor Celecoxib (25 mg/kg/day, forced feeding once a day, 21 days).

The procedure followed in the care and euthanasia of the study animals was in accordance with the European Community Standards on the Care and Use of Laboratory Animals (Ministère de l’Agriculture, France, authorization No. 6422).

**PHARMACOLOGICAL PROFILE OF THE ARTERIES**

First order mesenteric arteries were isolated and mounted on a wire-myograph (DMT, Aarhus, Denmark), as previously described (Henrion et al., 1992; Loufrani et al., 2002). Briefly, two tungsten wires (25 μm diameter) were inserted in the lumen of the arteries and fixed to a force transducer and a micrometer, respectively. A wall tension equivalent to a pressure of 100 mmHg was then applied. Arteries were bathed in a physiological salt solution (PSS) maintained at a pH of 7.4, a PO2 of 160 mmHg and a PCO2 of 37 mmHg. Cumulative concentration–response curves to acetylcholine were contracted with phenylephrine (1–3 μmol/L in order to reach 50% of maximal contraction) (Loufrani et al., 2002). Cumulative concentration–response curves were repeated after addition of one of the following agent: the NO-synthase blocker L-NAME (100 μmol/L), the non-specific COX inhibitor indomethacin (10 μmol/L), the COX2 inhibitor NS398 (10 μmol/L) or the TXA2/PGF2α (TP) receptor antagonist SQ29548 (10 μmol/L) (Racz et al., 2009). Endothelium-independent vasorelaxation was assessed with a CRC to sodium nitroprusside (SNP). Contraction to phenylephrine (1 nmol/L to 10 μmol/L) and to U46619 (stable Txα, mimetic, 1 nmol/L to 10 μmol/L) (Bolla et al., 2002) was assessed on a different arterial segment.

**WESTERN BLOT ANALYSIS**

Arterial segments were homogenized and proteins (25 μg total protein from each sample) were separated by SDS-PAGE using a 4% stacking gel followed by a 10% running gel. Proteins were detected with specific antibodies directed against eNOS (Transduction Laboratories) 1:1000 or COX2 (1:500, Santa Cruz Biotechnology) in TBST. Protein expression was visualized using the ECL-Plus Chemiluminescence kit (Amersham) (Belin de Chantemele et al., 2009).

**IMMUNOHISTOLOGICAL DETECTION OF COX2**

Cyclooxygenase-2 immunolabeling was performed as previously described (Henrion et al., 1997; Retailleau et al., 2010). Briefly, segments of mesenteric arteries were mounted in embedding medium (Tissu-Tek, Miles, Inc.), frozen in isopentane pre-cooled in liquid nitrogen, and stored at −80°C. Primary goat anti-COX2 polyclonal antibodies (1/200, Santa Cruz Biotechnology) were applied on the cross-sections (7 μm thick) followed by the fluorescent secondary antibody (1/200, Fluoroprobes). In negative control experiments the primary antibody was omitted. Positive control experiments were performed using mesenteric arteries from lipopolysaccharide-treated rats (Belin de Chantemele et al., 2010). Positive staining was visualized using confocal microscopy and QED-Image software (Solamere Technology). Image analysis was performed using Histolab (Microvision). The auto-fluorescent internal and external laminas, excluded for the fluorescence quantification, were used to delimit the media. Care was taken to take all the pictures in the same condition of laser power, gain, and exposure time.

**BLOOD PROSTANOIDS MEASUREMENT**

Blood samples were withdrawn from the femoral artery and placed in ice-cold polypropylene tubes containing EDTA plus indomethacin and centrifuged immediately at 3000 rpm for 15 min at 4°C and then stored at −80°C. Plasma samples were acidic with glacial acetic acid to pH 3 and then applied to octadecylsil (ODS) silica columns, washed with 15% aqueous ethanol and petroleum ether. The prostanoids were eluted with methyl formate. The eluent was evaporated to dryness under vacuum and the residue was dissolved in 400 L assay buffer. Thromboxane B2, 6-keto-PGF1α, alpha, and 8-isoprostane concentrations were measured using commercially available kits (Cayman Chemical). Hydrolysis with KOH 15% (40°C, 60 min followed by KH2PO4, 1 M) was performed before 8-isoprostane measurement). The total prostanoids concentration was measured as pg/mL.

**HISTOLOGICAL ANALYSIS**

In order to determine the cross-sectional area of mesenteric arteries, blood vessels were dissected under a microscope, mounted between two glass micropipettes and submitted to intraluminal pressure (100 mmHg). Arteries were bathed in a calcium-free PSS containing SNP (10 μmol/L). Arteries were then fixed in CARSON solution and embedded in Epon E812 resin. Semi-thin sections were stained with toluidine blue and observed under a DMR microscope (Leica). Image analysis was performed as previously described using the Histolab software (Microvision, Paris, France) (Belin de Chantemele et al., 2009).

**STATISTICAL ANALYSIS**

Results are expressed as mean ± SEM. Significance of the difference between arteries was determined by ANOVA (one-factor ANOVA or ANOVA for consecutive measurements, when appropriate). Mean values were compared by unpaired t-test or by the Bonferroni test for multigroup comparisons. Values of P < 0.05 were considered to be significant.
RESULTS

PHYSIOLOGICAL PARAMETERS

Rat body weight was significantly higher in obese Zucker rats than in lean rats, independent of age, and body weight was higher in 12-month-old rats than in young rats (Table 1). Mean arterial blood pressure was not affected by aging in lean rats or by metabolic syndrome in young animals. Nevertheless, mean arterial pressure was slightly, but significantly, increased in old obese Zucker rats compared to young or old lean animals. Blood glucose was not significantly affected by aging in lean rats and by metabolic syndrome in young animals. However glyceremia was significantly increased in old obese Zucker rats compared to young or lean rats (Table 1).

ENDOTHELIUM FUNCTION

Acetylcholine induced a concentration-dependent relaxation in isolated mesenteric arteries. Acetylcholine-mediated relaxation was significantly reduced in obese Zucker rats compared to lean rats and the combination of metabolic syndrome and aging further impaired endothelium-dependent relaxation (Figures 1A, B).

NO-synthesis inhibition with L-NAME reduced acetylcholine-mediated relaxation. Aging did not significantly affect the inhibitory effect of L-NAME in lean rats. On the other hand, in both young and old obese Zucker rats the inhibitory effect of L-NAME was significantly reduced compared to lean rats (Figure 1B). Indeed, acetylcholine-mediated maximal relaxation was inhibited by 78 ± 7% in young lean rats (Figure 1C) and by 49 ± 6% in young obese Zucker rats. L-NAME inhibited the relaxation by 75 ± 7% in old lean rats and by 56 ± 6% in old obese Zucker rats (P < 0.05 versus lean).

Indomethacin did not significantly affect acetylcholine-mediated relaxation in young obese Zucker and lean rats (Figure 1A). On the other hand, indomethacin further reduced acetylcholine-mediated vasorelaxation in old lean animals (Figure 1B), whereas indomethacin increased acetylcholine-mediated vasorelaxation in old obese Zucker rats (Figure 1B).

Precontraction prior to the addition of acetylcholine was similar in the four groups (Figure 1D).

The endothelium dysfunction associated with metabolic syndrome, aging, or the combination of aging plus metabolic syndrome was not associated with a change in eNOS expression level (Figure 1E).

Endothelium-independent relaxation (SNP) was not affected by aging or by metabolic syndrome (Figure 2A).

CONTRACTILITY

Contraction to phenylephrine was not affected by aging or chronic COX2 inhibition (data not shown) whereas contraction to U46619 was significantly higher in 12-month-old obese Zucker rats (maximal contraction: 16 ± 1.6 mN, n = 8) than in 12-month-old lean rats (11 ± 1.2 mN, n = 8) or than in 3-month-old rats (10 ± 1.4 mN in lean rats and 12 ± 1.4 mN in obese rats, n = 10 per group). In 12-month-old lean rats chronically treated with Celecoxib, U46619-induced contraction was significantly reduced (11 ± 1.5 mN, n = 8). The EC50 for U46619 was not affected by aging or metabolic syndrome (data not shown).

VASCULAR REMODELING

Arterial (luminal) diameter was reduced in obese Zucker rats compared to lean rats (significant in young animals only) and increased in old compared to young rats (Figure 2B). By contrast, media cross-section was higher in obese Zucker rats than in lean rats (again, significant in young animals only) and higher in old than in young rats (Figure 2C). Media to lumen ratio was significantly higher in old Zucker obese rats compared to lean animals (Figure 2D). In celecoxib-treated old rats media to lumen ratio remained significantly higher in old Zucker obese rats compared to lean animals (0.94 ± 0.11 in obese Zucker rats versus 0.63 ± 0.09 in lean rats, n = 8 per group, P < 0.05).

ROLE OF COX2 DERIVED PROSTANOIDS IN ENDOTHELIUM-MEDIATED RELAXATION

Acute COX2 inhibition with NS398 did not affect acetylcholine-mediated relaxation in young lean and obese Zucker rats but significantly improved acetylcholine-mediated relaxation in old obese Zucker rats (Figure 3A). No effect of NS398 was observed in old lean rats (Figure 3A).

TP receptor inhibition with SQ29548 did not affect acetylcholine-mediated vasorelaxation in young lean and obese Zucker rats while it significantly improved endothelium-dependent relaxation in old obese Zucker rats (Figure 3B).

In old obese Zucker rats plasma TxB2 (stable metabolite of TxA2), 6-keto-PGF alpha (stable metabolite of PGI2) and 8-isoprostane were significantly higher than in young rats (lean or obese) and than in old lean rats (Figure 4). In young obese Zucker rats chronically treated with Celecoxib plasma TxB2 and 6-keto-PGF alpha was reduced to the level found in lean animals. The treatment did not significantly change blood 8-isoprostane level. In young obese Zucker rats blood TxB2 was not significantly higher than in young lean rats (Figure 4).

Table 1 | Body weight, mean arterial pressure, and blood glucose measured in lean and obese Zucker rats aged 4 or 12 months.

| Age (months) | Lean rats | Obese Zucker rats |
|--------------|-----------|-------------------|
| 4            | 335 ± 11  | 388 ± 14*         |
| Mean arterial pressure (mmHg) | 97 ± 3    | 103 ± 4           |
| Blood glucose (mmol/L) | 8.6 ± 0.7 | 8.99 ± 0.9        |

| VEHICLE-TREATED RATS |
|----------------------|
| Body weight (g)      | 335 ± 11 | 388 ± 14* |
| Mean arterial pressure (mmHg) | 97 ± 3 | 103 ± 4 |
| Blood glucose (mmol/L) | 8.6 ± 0.7 | 8.99 ± 0.9 |

| CELECOXIB-TREATED RATS |
|------------------------|
| Body weight (g)        | 391 ± 12* | 696 ± 34** |
| Mean arterial pressure (mmHg) | 101 ± 3* | 126 ± 5** |
| Blood glucose (mmol/L) | 8.2 ± 1.0 | 13.11 ± 12** |

Rats were treated with the COX2 inhibitor Celecoxib or not (mean ± SEM, n = 8 per group).

*P < 0.05, young or old obese Zucker versus young lean rats.

**P < 0.05, old obese Zucker rats versus old lean rats.
Figure 1 | Acetylcholine-mediated relaxation in mesenteric resistance arteries isolated from young (A) or old (B) lean and obese Zucker rats. Concentration-response curves to acetylcholine were repeated after incubation with L-NAME (LN) and then with L-NAME plus indomethacin (LN + indo). Bar graphs (C) represent the maximal relaxation obtained in each group (taken from A and B). (D) Precontraction applied to the arteries before adding acetylcholine. (E) eNOS and β-actin expression level determined using Western blot. Mean ± SEM is presented (n = 8 per group). *P < 0.01, old versus young rats. #P < 0.01, obese versus lean rats. $P < 0.01, indo versus LN.
The novel finding of the present study is that the combined effect of metabolic syndrome and aging in a rat model, the obese Zucker rat, further impaired endothelium-dependent dilation due to COX2-dependent production of TxA2.

Endothelium-mediated relaxation was lower in old rats, especially in old obese Zucker rats, in agreement with previous studies although they were performed in young obese Zucker rats (Frisbee and Stepp, 2001; Katakam et al., 2005; Bouvet et al., 2007) or old control, or lean, rats (Ishida et al., 2003;...
Similarly, acetylcholine-mediated relaxation is reduced in 7-month-old obese Zucker rats compared to 5-month-old animals (Subramanian and MacLeod, 2003). We also found a reduction in L-NAME sensitive endothelium-mediated relaxation in both young and old obese Zucker rats. Although L-NAME blocks selectively NO-dependent dilation, this reduction was not associated with a change in eNOS expression level. This is in agreement with previous works performed in 2-year-old rats (Yan et al., 2007; Dumont et al., 2008) and in young obese Zucker rats (Karagiannis et al., 2003; Bouvet et al., 2007). Vascular smooth muscle cells response to the NO donor SNP was similar in the different groups, in agreement with previous studies (Steinberg et al., 1996; Oltman et al., 2005, 2006; Dumont et al., 2008). Thus observation suggests that vascular smooth muscle cells sensitivity to NO was not affected by aging or metabolic syndrome.

In lean old rats, part of the dilation was sensitive to indomethacin, suggesting the involvement of vasodilator prostanoids, in agreement with a previous study in older rats (Heymes et al., 2000). Relaxation resistant to L-NAME and indomethacin in young rats and in old obese Zucker rats was probably due to EDHF as previously shown in various vascular territories (Feletou and Vanhoutte, 2009). In addition, EDHF-dependent relaxation is reduced in obese Zucker mesenteric arteries (Young et al., 2008). Nevertheless, this issue was not investigated in the present study which focused on the involvement of COX-derived prostanoids.

**Figure 3** | Effect of COX2 inhibition with NS398 (A) or TxA2/PGH2 receptor blockade with SQ29548 (B) on acetylcholine-mediated relaxation in mesenteric resistance arteries isolated from young (A) or old (B) lean and obese Zucker rats. Mean ± SEM is presented (n = 8 per group). * P < 0.01, effect of NS398 (A) or SQ29548 (B).
In old obese Zucker rats indomethacin improved acetylcholine-mediated relaxation, suggesting the involvement of vasoconstrictor prostanoids. This vasoconstrictor prostanoid is most likely TxA$_2$ produced by COX2 as COX2 inhibition with NS398 and TxA$_2$/PGH$_2$ receptor blockade with SQ29548 similarly improved relaxation. In addition, contraction induced by the TxA$_2$ mimetic U49619 was higher in old obese Zucker rats whereas phenylephrine-induced contraction was not affected. The relationship between COX2, adrenergic receptor and TP receptors remains to be further investigated. Our findings are in agreement with previous reports in old rats (Briones et al., 2005; Gendron et al., 2007; Kang et al., 2007) and in both young (Dey et al., 2004) and old obese rats (Gendron et al., 2007) showing the involvement of COX2-derived vasoconstrictor agents in old rats. Similarly, TxA$_2$ impairs endothelium-mediated dilation.
Old obese Zucker rat were slightly, but significantly hyperglycemic and hypertensive at the age of 12 months (Table 1), in agreement with previous studies (Oltman et al., 2005). Nevertheless, hyperglycemia in old Zucker rats (+30%) was much lower than in Zucker diabetic rats (3- to 4-fold increase) (Oltman et al., 2005). Hypertension in old obese Zucker was associated with a significant increase in the media to lumen ratio, an index of wall hypertrophy. This was not observed in young rats. Although it is tempting to link the rise in blood pressure found in old obese Zucker rats excessive TxA2 production, as previously shown (Matrougui et al., 1997, 2000), hypertension in old obese Zucker rats was not reduced by the chronic blockade of COX2. Several studies have shown that hyperglycemia is associated with an excessive production of COX2-derived vasoconstrictor agents (Bagi et al., 2005; Matsumoto et al., 2007). Thus hyperglycemia, elevated in old obese Zucker rats, might be the cause, at least in part, of the induction of COX2 expression. Nevertheless, this issue remains...
to be further confirmed. Furthermore, the high oxidative stress found in old obese Zucker rats, evidenced by elevated blood level of 8-isoprostane, might also induce COX2 expression (Xiang et al., 2006, 2008).

The current study predicts a worsening of microvascular regulation in metabolic syndrome associated with age due to COX2-derived TxA2. Although, a chronic treatment of the rats with the COX2 inhibitor Celecoxib restored the relaxation to control level, COX2 inhibitors possess cardiovascular toxicity (Krotch et al., 2005; Dajani and Islam, 2008; Rao and Knaus, 2008).

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In conclusion we found that COX2-derived TxA2 reduced endothelium-mediated relaxation in mesenteric arteries of old obese Zucker rats, a model of metabolic syndrome.

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