

Proteinase-Activated Receptor-2 Mediates Arterial Vasodilation in Diabetes

Fiorentina Roviezzo, Mariarosaria Bucci, Vincenzo Brancaleone, Annarita Di Lorenzo, Pierangelo Geppetti, Silvana Farneti, Luca Parente, Giuseppe Lungarella, Stefano Fiorucci, Giuseppe Cirino

Objective—Proteinase-activated receptor-2 is widely expressed in vascular tissue and in highly vascularized organs in humans and other species. Its activation mainly causes endothelium-dependent vasorelaxation in vitro and hypotension in vivo. Here, using nonobese diabetic (NOD) mice at different disease stages, we have evaluated the role of PAR₂ in the arterial vascular response during diabetes progression.

Methods and Results—High (NOD-II; 20 to 500 mg/dL) or severe glycosuria (NOD-III; 500 to 1000 mg/dL) provokes a progressive reduction in the response to acetylcholine paralleled by an increase in the vasodilatory response to PAR₂ stimulation. Western blot and quantitative real-time polymerase chain reaction (RT-PCR) studies showed that this effect is tied to an increased expression of PAR₂ coupled to cyclooxygenase-2 expression. Pharmacological dissection performed with specific inhibitors confirmed the functional involvement of cyclooxygenase-2 in PAR₂ vasodilatory effect. This vasodilatory response was confirmed to be dependent on expression of PAR₂ in the smooth muscle component by immunohistochemistry studies performed on aorta isolated by both NOD-III and transgenic PAR₂ mice.

Conclusions—Our data demonstrate an important role for PAR₂ in modulating vascular arterial response in diabetes and suggest that this receptor could represent an useful therapeutic target. (*Arterioscler Thromb Vasc Biol.* 2005;25:2349-2354.)

Key Words: cyclooxygenase-2 ■ diabetes, type I ■ proteinase-activated receptor-2 ■ smooth muscle

Proteinase-activated receptors (PARs) are a recently described novel family of 7-transmembrane G-protein-coupled receptors.^{1–3} PARs are activated enzymatically through proteolysis of the receptor by a well-characterized family of enzymes that require serine within active site, the serine proteases. On cleavage at these specific sites, the new N-terminal for each receptor functions as a tethered ligand.² At the present stage, 4 subtypes PARs have been characterized: PAR₁,⁴ PAR₃,⁵ and PAR₄,⁶ selectively activated by thrombin and PAR₂ that is activated by trypsin, tryptase, tissue factor, factor VII, and factor X.¹

PAR₂ has been shown to be involved in cardiovascular function.^{7,8} Functional studies have shown that activation of the receptor by using a small agonist peptide derived from the tethered ligand sequence (PAR₂AP) causes an endothelium-dependent vasodilatation.^{9–13} Subsequent studies have clearly shown that PAR₂ is expressed in the human endothelial¹⁴ and smooth muscle cells.¹⁵ Systemic administration of PAR₂AP in vivo in rats and mice causes hypotension.^{10,11,16,17} Interestingly, also in humans in vivo PAR₂ AP causes vasodilatation that is dependent on nitric oxide (NO) release and prostanoids.¹⁸ As opposed to PAR₁, PAR₂ expression is upregulated

by inflammatory stimuli such as tumor necrosis factor- α , bacterial endotoxin (lipopolysaccharide [LPS]) and IL-1 α in endothelial cells.^{16,19,20} Likewise, LPS administration to rats increases PAR₂ expression on endothelium and smooth muscle cells from both jugular vein and carotid artery, which translates in an increased vasodilatory response to PAR₂ AP.¹⁶ Consistent with these findings, the dose necessary to activate the receptor in several inflammatory conditions is reduced to 1 or 2 orders of magnitude in comparison to normal conditions. Whereas all these data suggest a role for PAR₂ in cardiovascular inflammation, it is unclear whether the activation of this receptor plays a pathological or compensatory role.^{20,21}

One example of endothelial dysfunction coupled to cardiovascular inflammation is represented by diabetes. Duration of diabetes and poor glycemic control are the main predictor factors for peripheral arterial disease, indicating a close link between metabolic impairment and arterial injury.^{22,23} Although peripheral arterial disease results from atherosclerotic narrowing of the blood vessel lumen, endothelial dysfunction has been shown to play a role in the progression of this disease. Further, these processes are enhanced by the meta-

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From Dipartimento di Farmacologia Sperimentale (F.R., M.B., V.B., A.D.L., G.C.), Università di Napoli Federico II; Dipartimento di Scienze Farmaceutiche (L.P.), Università di Salerno; Dipartimento di Aria Critica Medico Chirurgica (P.G.), Università di Firenze; Dipartimento di Medicina Sperimentale (S.F., S.Fiorucci), Università di Perugia; Dipartimento di Fisiopatologia e Medicina Sperimentale (G.L.), Università di Siena.

Correspondence to Giuseppe Cirino, PhD, Dipartimento di Farmacologia Sperimentale, via Domenico Montesano 49 80131 Napoli, Italy. E-mail cirino@unina.it

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bolic disturbances associated with diabetes and denervation of the smooth muscle of the tunica media of arteries caused by the diabetic neuropathy. Whether PAR₂ plays a role in the pathogenesis of arterial dysfunction in diabetes and if it is upregulated in this condition are unknown.

In the present study, by using a genetic mouse model of type I diabetes and PAR₂ transgenic mice, we have investigated the role of PAR₂ in vessel function during the disease progression. The results of our studies indicate that arterial expression of PAR₂ increases in response to diseases progression and exerts a potent vasorelaxant effect, suggesting that development of PAR₂ agonist might be helpful in treating patients with diabetic arthropathy.

Materials and Methods

Animals

Female nonobese diabetic mice (NOD/Ltj) and CD-1 mice were purchased from Charles River (Italy). CD-1 mice were used as control animal because they display a similar pattern of contraction and relaxation to NOD-I (Figure 1, available online at <http://atvb.ahajournals.org>). Transgenic PAR₂ (tg-PAR₂) mice were bred at University of Siena and have been previously described.²⁴ NOD mice were divided according to the glycosuria value (Figure II, available online at <http://atvb.ahajournals.org>) in the following groups: NOD-I: low or null glycosuria (5±2 weeks; 0 to 20 mg/dL); NOD-II: high glycosuria (13±3; 20 to 500 mg/dL); and NOD-III severe glycosuria (22±3; 500 to 1000 mg/dL). For further details, see <http://atvb.ahajournals.org>.

In Vitro Experimental Protocols

In each experiment, rings were standardized using phenylephrine (1 μmol/L) until the responses were reproducible. To evaluate tissue vasorelaxation, cumulative concentration response curve to Ach (10 nmol to 30 μmol/L), and to the PAR₂ tethered ligand peptide (10 nmol to 30 μmol/L) were performed on 5HT (3×10⁻⁷ mol) precontracted rings. Curves to PAR₂AP were constructed in the absence and presence of L-NAME (100 μmol/L, 20 minutes), 1400 W (10 μmol/L), ibuprofen (10 μmol/L), DFP (10 μmol/L), FR-122047 (20 μmol/L), and SQ-22,536 (100 μmol/L). When L-NAME was added, a submaximal dose (EC₅₀) of 5-HT (3×10⁻⁸ mol) was used.

Western Blotting

Western blotting studies were performed on aortic tissue samples homogenized in lysis buffer using a Talon homogenizer and were processed identically. The immunoblots were developed with 1:500 dilutions for PAR₂ and 1:1000 for COX-2 and the signal was detected with the ECL System according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Quantitative Real-Time Polymerase Chain Reaction Studies

After the mice had been killed, aortas were removed and immediately snap-frozen on liquid nitrogen (LN₂) and stored at -80°C until used. Total RNA was isolated using TRIzol reagent (Life Technologies, Milan, Italy) as previously described.^{26,27} Quantification of the expression mouse genes was performed by quantitative real-time polymerase chain reaction (RT-PCR) by using specific primers.

Immunohistochemistry

The aortas from the different groups of mice were fixed with buffered formalin (5%) for 24 hours. Tissue sections (6 μm) were stained for PAR₂ receptors by an immunoperoxidase method. The primary polyclonal antibody (Ab) used was a goat polyclonal Ab raised against the carboxyl terminus of PAR₂ of human origin, and the specificity of the antibody was tested using the blocking peptide (sc-8205P). The Ab was used at dilution of 1:200.

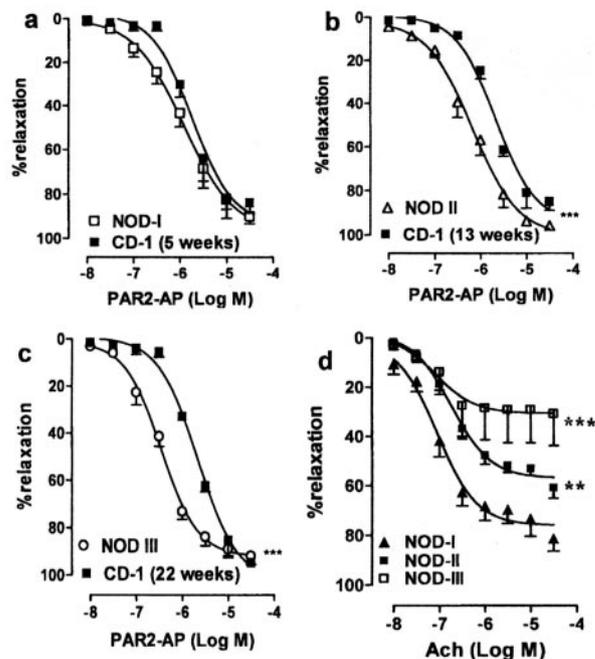


Figure 1. The vasorelaxant effect produced by PAR₂AP on NOD-I aorta (panel a; n=8) is not significantly different from that produced on CD-1 (5±2 weeks) aorta (n=8, a). The vasorelaxant effect is significantly potentiated in NOD-II aorta versus CD-1 (13±3 weeks) (n=8, b) and in NOD-III vs CD-1 (22±3 weeks) aorta (n=6, c). The disease progression causes a clear reduction in the acetylcholine-induced relaxation of NOD mouse isolated aortas (d); ****P*<0.001; ***P*<0.01; n represents the number of mice used from each animal were prepared at least 4 aorta rings.

Statistical Analysis

Data are expressed as mean±SEM. The level of statistical significance was determined by 1-way analysis of variance (ANOVA) followed by Bonferroni's *t*-test for multiple comparisons, using the GraphPad Prism software.

Results

Diabetes Progression Increases PAR₂AP-Induced Vasodilatation and Upregulates PAR₂ and COX-2 Expression

In NOD-I and CD-1 mice, that have normal glycosuria, the relaxant response induced by PAR₂AP on isolated aorta is similar (Figure 1a). The disease progression leads to a significant decrease in the relaxant response to acetylcholine (Figure 1d) with a concomitant increase in PAR₂AP-induced vasodilatation of NOD-II (Figure 1b) and NOD-III (Figure 1c) aortas. There is a significant shift of PAR₂AP-induced vasorelaxant effect of the EC₅₀ from 1×10⁻⁶ mol of NOD-I aorta to 6.5×10⁻⁷ for NOD-II aorta and to 4×10⁻⁷ mol/L for NOD-III aorta. The scramble peptide LSIGRL-NH₂ was inactive on both NOD-I and NOD-III mice aorta (Figure III, available online at <http://atvb.ahajournals.org>). Because it is known that inflammatory stimuli can upregulate both PAR₂ and COX-2 expression, we have evaluated expression of PAR₂ in aortas obtained from normal and NOD mice at different stages of illness (NOD-I, NOD-II, and NOD-III). Quantitative RT-PCR showed an increase in mRNA expression of PAR₂ (Figure 2A, panel f) and COX-2 (Figure 2A,

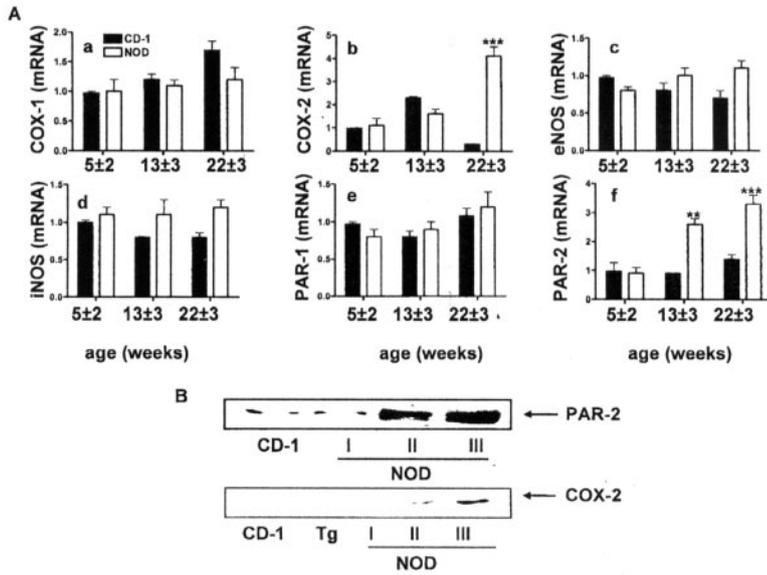


Figure 2. A, RT-PCR analysis shows that there is a significant increase in mRNA for PAR₂ (f), COX-2 (b) but not PAR₁ (e), eNOS (c), COX-1 (a), and iNOS (d) in aortas isolated from NOD-II and NOD-III mice **P*<0.05 vs their relative control. B, Western blot analysis shows PAR₂ and COX-2 protein expression. Analysis was performed on aortas from NOD-II, NOD-III, and tg-PAR-2 (Tg) mice. Experiments were performed in triplicate (n=3 mice).

panel b), whereas PAR₁ (Figure 2A panel e), COX-1 (Figure 2A, panel a), inducible nitric oxide synthase (iNOS) (Figure 2A, panel d), and endothelial nitric oxide synthase (eNOS) (Figure 2A, panel c) mRNA levels were unchanged. Western blot analysis showed that there is a concomitant significant increase of PAR₂ and COX-2 protein expression in NOD-II and NOD-III aortas when compared with either NOD-I or CD-1 aortas (Figure 2B), whereas iNOS was unchanged (data not shown).

Role of NO and Prostanoids in PAR₂AP-Induced Vasodilatation in NOD Mice

Next, to investigate on the role played by NO and prostanoids, we tested the effect of L-NAME and ibuprofen. Each drug was used at concentrations known to inhibit NO-dependent vasorelaxation and COX-1/COX-2 activity, respectively. Incubation of aorta rings of CD-1 (Figure 3a) or NOD-I (Figure 3b) with L-NAME abrogated the vasodilatory effect of PAR₂AP. Conversely, ibuprofen did not affect

PAR₂AP-induced vasodilatation of either CD-1 (Figure 3a) or NOD-I (Figure 3b) mouse aorta.

Aorta isolated from NOD-II mice show a reduced inhibitory effect of L-NAME that was removed at lower doses with a significant shift of E_{max} (maximal relaxation achievable) from 90% to 25% (Figure 3d). Similarly, there is a significant inhibition by ibuprofen (Figure 3d) particularly marked at lower doses of PAR₂AP. NOD-III isolated aortas displayed a similar inhibitory pattern with a more marked inhibitory effect displayed by ibuprofen (Figure 3e). When aortic rings were incubated with both ibuprofen and L-NAME, there was a further significant inhibition of the relaxant response to PAR₂AP. Prostaglandins produce their effect through cAMP. To further confirm cAMP involvement we used SQ-22,536 an inhibitor of adenylate cyclase. SQ-22,536 significantly reduced PAR₂AP-induced vasorelaxation of NOD-III aorta (Figure 3f), but it was ineffective in NOD-I aortas (Figure 3c). Functional studies performed on aortas isolated by CD-1 (Figure 4a), NOD-I (Figure 4b), and NOD-III (Figure 4c) by

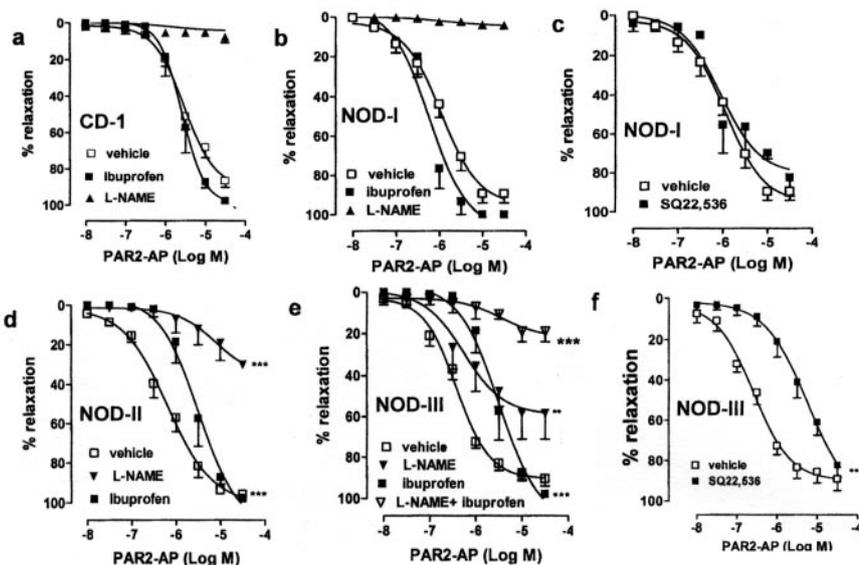


Figure 3. L-NAME (100 μmol/L) but not ibuprofen (10 μmol/L) significantly inhibits PAR₂AP-relaxation of aortas isolated by either CD1 (panel a; n=6) or NOD-I (panel b; n=6) mice. Both NOD-II (panel d; n=6) and NOD-III (panel e; n=4) mouse aortas displayed a significant reduction in the L-NAME inhibitory effect and the appearance of a significant inhibitory effect of ibuprofen. Incubation with L-NAME and ibuprofen further inhibited the vasorelaxant response (panel e). SQ-22,536 (100 μmol/L), an inhibitor of adenylate cyclase did not modify the PAR₂AP induced vasorelaxant response in NOD-I mice (panel c; n=6) but strongly inhibited PAR₂AP-induced relaxation in NOD-III mouse aorta (panel f; n=5). Data are expressed as mean±SEM. ***P*<0.01, ****P*<0.001 vs vehicle; n represents the number of mice used, from each animal were prepared at least 4 aorta rings.

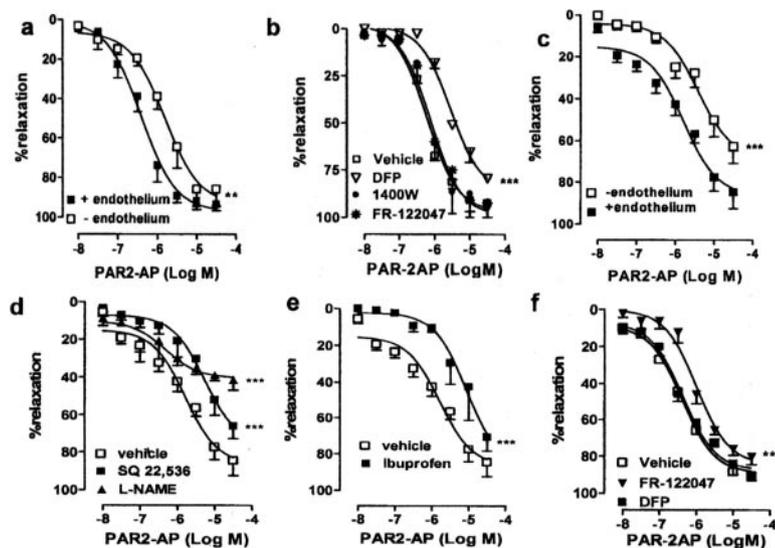


Figure 4. Endothelium mechanical removal from NOD-III (n=5; a) and tg-PAR₂ (n=4; c) mouse aorta does not abrogate PAR₂AP relaxation. PAR₂AP-induced relaxation of NOD-III mouse aorta is significantly inhibited by DFP only (n=3; b). PAR₂AP-induced relaxation of tg-PAR₂ mouse aorta is significantly inhibited by ibuprofen (n=4; e), FR-122047 (n=4; f) and SQ-22,536 (n=4; d) and to a lesser extent by L-NAME (n=4; d), whereas DFP (n=3; f) has no effect. Data are expressed as mean ± SEM. ****P*<0.001, ***P*<0.01; n represents the number of mice used from each animal were prepared at least 4 aorta rings.

using specific inhibitors of COX-1 (FR-122047), COX-2 (DFP), and iNOS (1400W) showed that the major contribute to the relaxation observed in NOD-III mice is given by prostanoids mainly derived by COX-2. These data fit well with the increased COX-2 expression observed in NOD-II and NOD-III mouse aortas (Figure 2). Aorta harvested from CD-1 mice age-matched with NOD mice did not show any change in relaxation induced by acetylcholine or PAR₂AP; similarly, glycosuria was unchanged (Figure 1).

Tg-PAR₂ Mice

To confirm these data, we used transgenic mice overexpressing PAR₂. The vasorelaxant response of NOD-III aorta to PAR₂AP (Figure 4a) is similar to that of tg-PAR₂ mouse isolated aorta (Figure 4c). Furthermore, similarly to what happens in NOD-III aortas (Figure 4a), PAR₂AP-induced vasorelaxation is still present in endothelium-denuded aortic rings obtained by tg-PAR₂ mice (Figure 4c). These data clearly suggest an active role of smooth muscle cells in PAR₂-mediated vasorelaxation in tg-PAR₂ mice, suggesting that a similar increase in PAR₂ expression in the smooth muscle component of NOD-II and NOD-III mice. We therefore evaluated the expression of PAR₂ in situ by using immunohistochemical staining of aortas obtained from wild-type and NOD-III mice, as well as from transgenic overexpressing PAR₂ (Figure 5). As shown in Figure 5, PAR₂ expression was minimal but nonetheless detectable in a patchy distribution in aorta and peri-aortal tissue of wild-type mice with very low positive reaction on the smooth cell component (Figure 5, panel A). By contrast, expression of PAR₂ was diffusely greater in tissues of NOD mice (Figure 5, panel B). The specific staining for PAR₂ we observed on the smooth muscle cell component in aortas from NOD-III mice (Figure 5, panel B) equaled, or exceeded, in strength that of aortas from mice overexpressing PAR₂ (Figure 5, panel C). The positive staining was removed by addition of the blocking peptide confirming the specificity of the primary antibody (Figure 5, panel D). Thus, the immunohistochemistry data support the functional data. Next, to further address this similarity between NOD-III and tg-PAR₂ mice, we performed

a comparative study using ibuprofen, L-NAME, SQ-22,536, DFP, and FR-122047. Similar to what happens in NOD-III mice, ibuprofen (Figure 4e) and SQ-22,536 (Figure 4d) both inhibited PAR₂AP-induced vasorelaxation in intact rings harvested from tg-PAR₂ mice. As in NOD-III mice, L-NAME inhibitory effect on tg-PAR₂ mouse aorta was reduced as Emax (Figure 4d). DFP, the selective COX-2, inhibitor significantly reduced PAR₂AP-induced relaxation in NOD-III aorta (Figure 4b). Conversely, the selective COX-1 inhibitor (FR-122047) that was ineffective in NOD-III mice significantly inhibited PAR₂AP-induced vasorelaxation in tg-PAR₂ mice whether DFP had no effect (Figure 4f).

Discussion

Cardiovascular diseases are currently the principal causes of morbidity and mortality in patients with type I diabetes.^{22,23} The loss of modulator tone by the endothelium is considered one of the critical factor in the development of diabetic vascular diseases. There is evidence that an impaired response to endothelium-dependent agonists in different vascular beds develops in response to both chemically induced and

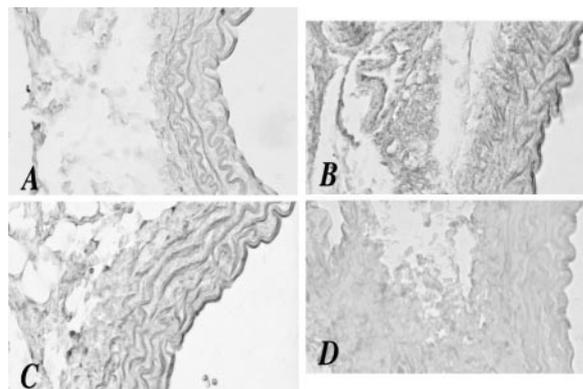


Figure 5. Immunohistochemical staining for PAR₂ in aortas from wild-type mouse (A), NOD III (B), and tg-PAR₂ mice (C). The positive staining was removed by addition of the blocking peptide confirming the specificity of the primary antibody (D). Original magnification (100×) shows a clear intense staining for the smooth muscle component and of the peri-aortal area.

genetic models of type I diabetes or in vivo in experimental animals.²⁸ A constant feature of these studies is an impaired response to acetylcholine. We have recently shown that NOD mice vessel reactivity follows the diabetes progression and, in particular, there is an impairment of the vasorelaxant effect to acetylcholine and isoprenaline.^{25,29}

Here, we show that response to PAR₂ selective stimulation is increased in diabetic mice with a clear shift of the EC₅₀. This increased response is strictly linked to the disease progression being maximal in NOD-III mice in which vasodilatation to acetylcholine is strongly impaired through an alteration of the post-translational mechanisms involved in eNOS regulation.²⁵ The vasorelaxant effect of PAR₂ on vessels is linked to the presence of an intact endothelium and only in part through the NO release as demonstrated both in vitro and in vivo.^{7,10,11,30} Recently it has been shown that PAR₂ activation increases COX-2 protein and mRNA expression and promotes PGI₂ release from human umbilical vein endothelial cell. Thus, COX-2 induction takes part in the functional response of endothelial cells to PAR₂ activation,³¹ suggesting that PAR₂ promotes a sustained upregulation of prostanoid production in endothelium. To gain further insights into this mechanism we have analyzed the effect of L-NAME, an NO synthase inhibitor, and ibuprofen, a COX-1/2 inhibitor. In control and NOD-I mice (low or null glycosuria), L-NAME virtually abrogates the PAR₂AP induced vasorelaxation whereas ibuprofen was ineffective. Conversely, when diabetes is clearly established, such as in NOD-II mice, there is a reduction in the inhibitory effect of L-NAME and the appearance of ibuprofen inhibitory effect on PAR₂AP-induced relaxation, which is particularly evident at the lower doses of PAR₂AP. In NOD-III mice, that have a more severe glycosuria, there is equally a loss in efficacy of L-NAME and the inhibitory effect of ibuprofen is significantly more pronounced. The simultaneous administration of ibuprofen and L-NAME further inhibited the relaxant response to PAR-2AP. These results imply that, as the disease progresses, there is a gradual switch of the vessel relaxant mechanism toward the PAR₂ signaling pathway with an increased contribute of the cyclooxygenase pathway. Functional studies performed using selective inhibitors of COX-1 (FR-122047) and COX-2 (DFP) confirmed that PAR-2AP induced vasorelaxation occurs with a COX-independent mechanism in control condition, eg, in NOD-I and CD1 mouse aortas. Conversely, in pathological condition, such as in NOD-III mice, there is a clear involvement of prostanoids, mainly driven by COX-2 as suggested by the lack of inhibitory effect of the selective COX-1 inhibitor FR-122047 and by the marked effect of SQ22,536, a selective cAMP inhibitor. This interpretation is supported by the molecular studies that clearly show by quantitative RT-PCR an increase in PAR₂ and COX-2 mRNAs expression that correlates well with protein expression in NOD-III mice. Interestingly, the immunohistochemistry study clearly demonstrated that diabetes development causes an increased expression of PAR₂ on the smooth muscle cell component and in peri-aortic areas. Thus, it appears that there is a linkage among NO, PAR₂, and COX-2 that becomes evident in pathological condition. A similar linkage among PAR₂AP vasodilatory effect, NO, and

COX products has been recently shown also in human volunteers¹⁸ and in experimental animals.³⁰ Using rat aorta has been shown that basal NO modulates the vascular effects linked to PAR₂ activation and that both cGMP and cAMP are involved.³⁰ In human healthy volunteers it has been shown that vasodilatation of the dorsal hand vein induced by local administration of PAR₂AP is inhibited by both L-NAME and aspirin.¹⁶ In this context it is important to note that PAR₂, as opposed to PAR₁, can be upregulated by inflammatory stimuli such as tumor necrosis factor- α , IL-1 β , and LPS.¹⁶ This upregulation is also present in vivo after administration of LPS in the arterial and venous tissue of rats¹⁹ and in vitro in human coronary vessels.³² Similar results that further support the hypothesis that PAR₂ can be “unmasked” by an inflammatory cardiovascular event were obtained by using an animal model of balloon vascular injury.³³

Because in our vessel preparation there is still a residual activity to Ach, and to better-understand the role of endothelium in our experimental condition, we tested the effect of PAR₂AP in rings where the endothelium was mechanically removed. Endothelium removal in NOD-I mice comported a complete loss of the relaxing activity similar to what can be observed in CD-1 mice as it has been demonstrated also by others. Conversely, no significant changes were observed in NOD-III mice in relaxation in aorta after endothelium removal, furthermore the pattern of relaxation operated by PAR-2AP in vitro was very close to that displayed by aorta isolated by tg-PAR-2 mice. In a more complete analysis of the response to PAR₂AP of tg-PAR₂ aorta, the similarity with the response to NOD-III mice was even more striking. Similar to NOD-III isolated aortas, tg-PAR₂ mice isolated aortas displayed a reduced vasodilatory response to PAR₂AP in presence of ibuprofen and SQ-22,546 as well as reduced inhibition by L-NAME. Interestingly, the selective COX-1 inhibitor was ineffective on aortas isolated from NOD-III, whereas it significantly inhibited tg-PAR₂ mouse aorta, further supporting that the disease development causes a selective induction of the COX-2 isoform. Thus, these data support the hypothesis that after endothelial injury there is an increased expression of PAR₂ on the smooth muscle cells to counterbalance the loss in vasodilatory component. In NOD-III mice, the prostanoid effect is mainly driven by COX-2 as opposed to tg-PAR-2 mice, in which it is mainly driven by COX-1. This is not surprising because in NOD mice, the slow development of diabetes can cause the COX-2 induction, whereas in tg-PAR-2 mice the increased expression of PAR-2 is obtained by genetic manipulation and, for this reason, couples to the constitutive form of the enzyme, ie, COX-1.

In conclusion, our data show that in diabetes development there is a gradual switch of the vessel relaxant function toward PAR₂ and COX-2. This most likely represents a functional response to the injury to the endothelium that in this condition displays a reduced functionality. These data also suggest that PAR₂AP peptide may be useful vasodilator in diabetes or in other pathology in which an endothelial damage on inflammatory basis is present. In this context it has been recently demonstrated a beneficial effect of PAR₂AP in a murine model of hind-limb ischemia in which PAR₂AP

administration increases capillarity resulting in an accelerated hemodynamic recovery and enhanced limb rescue.³⁴

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Proteinase-Activated Receptor-2 Mediates Arterial Vasodilation in Diabetes

Fiorentina Roviezzo, Mariarosaria Bucci, Vincenzo Brancaleone, Annarita Di Lorenzo, Pierangelo Geppetti, Silvana Farneti, Luca Parente, Giuseppe Lungarella, Stefano Fiorucci and Giuseppe Cirino

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Materials and Methods

Reagents

L-phenylephrine (PE), acetylcholine (Ach), serotonin (5-HT), sodium nitroprusside (SNP), N^o-nitro-L-arginine methyl ester (L-NAME), ibuprofen, SQ-22,536, Trypsin (16,400U/mg) were purchased from Sigma Chemical Co. (Milano-Italy). DFP (5,5-dimethyl-3-(2-propoxy)-4-(4-methanesulfonylphenyl)-2(5H)-furanone) was a generous gift of Prof. TD Warner (UK). 1400W and FR-122047 were purchased from Tocris Cookson (Bristol UK). All salts used for Krebs solution preparation were purchased from Carlo Erba Reagenti (Milan, Italy). PAR₂ agonist peptide (SLIGRL-NH₂) and the scrambled peptide (LSIGRL-NH₂) were a generous gift of prof V.Santagada and G Caliendo (Napoli, Italy). Anti-PAR₂ IgG (sc-8205) and the blocking peptide (sc-8205P) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, California, USA). Anti-COX-2 and anti iNOS were purchased from Cayman-Chemical (USA). All salts used for western blot analysis were purchased from ICN Biochemical (Eschwege, Germany).

Animals

Female *Non Obese Diabetic mice* (NOD/Ltj) and CD-1 mice, were purchased from Charles River (Italy). CD-1 mice were used as control animal since they display a similar pattern of contraction and relaxation to NOD I (supplemental fig.1) NOD mice exhibit a susceptibility to spontaneous development of autoimmune (type I) insulin dependent diabetes mellitus (IDDM). Diabetes development in NOD mice is characterised by lymphocyte infiltration into the pancreatic islets which progressively induces pancreatic β cell necrosis. Progressive reduction in pancreatic insulin content starts at about 12-16 weeks of age. Transgenic PAR₂ (PAR₂tg) mice were bred at University of Siena and have been previously described²⁴. Briefly, a 140 Kb human BAC clone (identification no. 23C20) containing the entire genomic sequence of human PAR₂ was used. This BAC DNA (5ng/ μ l) was microinjected into the pronuclei of FVB/N eggs. FO pups were screened

for incorporation of the transgene using the human-specific PAR₂ primer and it was confirmed by Southern blotting.

NOD mice

To assess the diabetic condition of NOD animals, glycosuria was evaluated weekly to select the animals. This method was used since it is non invasive and it well correlates with an increase in blood glucose²⁵. The content of glucose in the urine was measured by using Trinder reaction (Glucose Trinder 100, Sigma Chemical Co. Milano, Italy). Mice were divided according to the glycosuria value (mg/dl) in the following groups: NOD- I: low or null glycosuria (5±2 weeks; 0-20 mg/dl) ; NOD-II: high glycosuria (13±3; 20-500 mg/dl) ; NOD-III severe glycosuria (22±3; 500-1000 mg/dl). The day of the sacrifice blood glucose levels were also measured. Animals were sacrificed according to the glycemia/glycosuria levels and aortas were dissected and used for western blotting analysis or for tissue bath experiments.

Tissue preparation.

NOD, tg-PAR₂ or CD-1 mice were sacrificed and thoracic aorta was rapidly dissected and cleaned from fat and connective tissue. Rings of 1.5-2 mm length were cut and mounted on wire myographs (Kent Instruments, Japan) filled with gassed Krebs solution (95% O₂+ 5% CO₂) at 37°C. Changes in isometric tension were recorded with PowerLab data acquisition system (Ugo Basile, Italy). The composition of the Krebs solution was as follows (mol/l): NaCl 0.118, KCl 0.0047, MgCl₂ 0.0012, KH₂PO₄ 0.0012, CaCl₂ 0.0025, NaHCO₃ 0.025 and glucose 0.010. Rings were initially stretched until a resting tension of 1.5 g was reached and allowed to equilibrate for at least 40 minutes during which tension was adjusted, when necessary, to 1.5 g and bathing solution was periodically changed. In a preliminary study a resting tension of 1.5 g was found to develop the optimal tension to stimulation with contracting agents.

In vitro experimental protocols.

In each experiment rings were firstly challenged with PE (1 μ M) until the responses were reproducible. Conversely, to evaluate tissue vasorelaxation, cumulative concentration response curve to Ach (10 nM-30 μ M), to trypsin (0.01-10U/ml) and to the PAR₂ tethered ligand peptide (10 nM-30 μ M) were performed on 5HT (3x10⁻⁷M)- precontracted rings. Percent of relaxation was calculated as follows: drug induced vasorelaxation (g) / 5-HT induced contraction (g) x100. In another set of experiments, rings were denuded of the endothelium and a PAR₂AP cumulative concentration-response curve was performed. To investigate the involvement of NO, prostanoids and cAMP, dose response curves to PAR₂AP were constructed in the absence and presence of either N^G-nitro-L-arginine methyl ester (L-NAME, 100 μ M, 20 minutes) to inhibit NOS, 1400W (10 μ M) to inhibit iNOS, cyclooxygenase inhibitor ibuprofen (10 μ M) to inhibit prostaglandin synthesis, DFP (10 μ M) to inhibit COX-2, FR-122047 (20 μ M) to inhibit COX-1, and SQ-22,536 (100 μ M) to inhibit adenylate cyclase. When L-NAME was added, a submaximal dose (EC₈₀) of 5-HT (3x10⁻⁸M) was used. When rings were incubated with L-NAME was reached an increase in tension in dyne/mg/wet tissue that did not significantly differ from that obtained using 5-HT at 3 x10⁻⁷ M alone (data non shown). The relaxation achieved at the higher dose tested is defined as Emax.

Western Blotting.

Aortic tissue samples were homogenised in lysis buffer (β -glycerophosphate 0.5 M, sodium orthovanadate 10mM, MgCl₂ 20mM, EGTA 10mM, DTT 100mM and protease inhibitors) using a Talon homogenizer, and were processed identically. Protein concentration was determined using Bradford assay (Bio-Rad Laboratories, Segrate, MI). Proteins (30 μ g) were subjected to electrophoresis on an SDS 10% polyacrylamide gel and electrophoretically transferred onto a nitrocellulose transfer membrane (Protran, Schleicher & Schuell, Germany). The immunoblots were developed with 1:500 dilutions for PAR₂ and 1:1000 for COX-2 and the signal was detected with the ECL System according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Quantitative RT-PCR

After the mice had been killed, aortas were removed and immediately snap-frozen on liquid nitrogen and stored at -80°C until used. Total RNA was isolated using TRIzol reagent (Life Technologies, Milan, Italy) as previously described^{26,27}. Quantification of the expression mouse genes was performed by qRT-PCR. The sense and antisense primers for COX-1, COX-2, PAR₁, PAR₂, iNOS and eNOS were obtained from Stratagene (La Jolla, CA;), Sigma Genosys (UK) or Maxim Biotech (San Francisco, CA). All PCR primers were designed using software PRIMER3-OUTPUT using published sequence data from the NCBI database. The RNA was reverse-transcribed with Superscript III (Invitrogen) in 20 μl reaction volume using random primers. For RT-PCR, 100 ng template was used in a 25 μl containing 0.3 μM of each primer and 12.5 μl of 2 x SYBR Green PCR Master mix (Bio-Rad). All reactions were performed in triplicate and the thermal cycling conditions were as follows: 2 min at 95°C , followed by 50 cycles of 95°C for 10 sec, and 60°C for 30 sec in an iCycler iQ instrument (Biorad, Hercules, CA). The mean value of the replicates for each sample was calculated and expressed as the cycle threshold (CT: cycle number at which each PCR reaction reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference between the CT value of the sample for the target gene and the mean CT value of that sample for the endogenous control (*GADPH*).

Immunohistochemistry.

The aortas from the different groups of mice were fixed with buffered formalin (5%) for 24 hours. The samples were then dehydrated, cleared in toluene, and embedded in paraffin. Tissue sections (6 μm) were stained for PAR₂ receptors by an immunoperoxidase method. The sections were pre-treated with 3% hydrogen peroxide to inhibit the activity of the endogenous peroxidase. For antigen

retrieval, the sections were heated in a microwave for 20 min in citrate buffer 0.01M, pH 6.0, and allowed to cool slowly to room temperature. All the sections were also incubated with 3% bovine serum albumin for 30 min at room temperature to block non-specific antibody binding. They were then incubated overnight at 4°C with the primary antibodies (Ab). The primary polyclonal Abs used was a goat polyclonal Ab raised against the carboxyl terminus of PAR₂ of human origin, and the specificity of the antibody was tested using the blocking peptide (sc-8205P) purchased from the same manufacturer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). This Ab cross-reacts with mouse PAR₂ and was used at dilution of 1:200. The sections were rinsed and incubated with sheep anti-goat IgG antibodies for 40 min at room temperature. The staining was revealed by adding peroxidase-antiperoxidase complex prepared from goat serum. Detection was accomplished by incubating in diaminobenzidine freshly dissolved in 0.03% H₂O₂ in 50 mM Tris-HCl pH 7.6. The sections were counterstained with haematoxylin.

Statistical Analysis.

Data are expressed as mean \pm s.e. mean. The level of statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni's *t*-test for multiple comparisons, using the GraphPad Prism software.

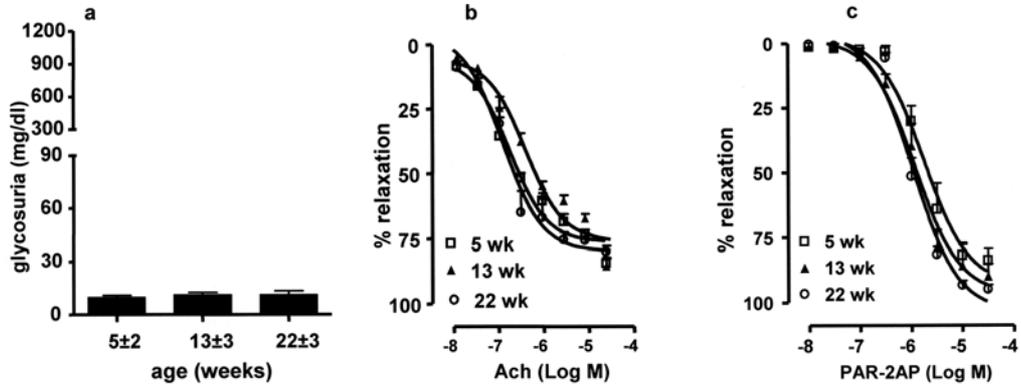


Figure 1

CD-1 mice do not show any age related increase in glycosuria (panel a). The vasorelaxant effect produced by Ach (panel b) and PAR2AP (panel c) in CD-1 aorta does not significantly differ among 5±2, 13±3 and 22±3 weeks old mice.

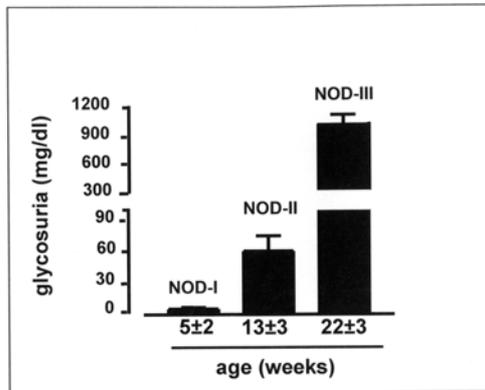


Figure II

Diabetes progression causes an increase in glycosuria that is related to age and disease progression (n= 5-8)

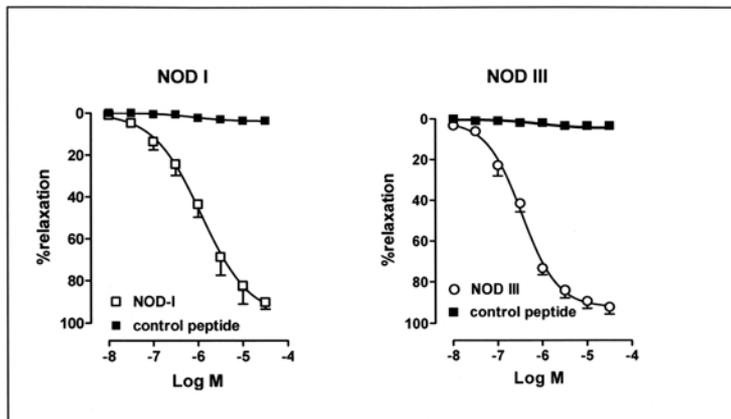


Figure III

The scramble peptide (LSIRGL-NH₂) has no effect on NOD I aorta (n=4) and NOD III (n=3) isolated aortas