Altered arachidonic acid-mediated responses in the perfused kidney of the streptozotocin-induced diabetic rat

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Abstract

Using perfused kidneys isolated from age-matched controls and streptozotocin (STZ)-induced diabetic rats, we investigated the effects of arachidonic acid (AA) on perfusion pressure in the presence of methoxamine. AA elicited a transient contraction followed by a sustained relaxation in each group. The amplitude of contraction was smaller in the diabetic group than in the control group, whereas the amplitude of the sustained relaxation was greater in the former than in the latter group. In the diabetic group, the AA-induced sustained relaxation was completely inhibited by indomethacin [cyclooxygenase (COX) inhibitor], SKF525A [cytochrome P450 (CYP450) inhibitor], or clotrimazole (epoxygenase inhibitor), but not by furegrelate [thromboxane A₂ (TXA₂)-synthase inhibitor], SQ29548 (TXA₂-receptor antagonist), or baicalein [lipoxygenase (LOX) inhibitor]. In the diabetic kidney, more-or-less additive inhibitions of the AA-induced relaxation were seen when indomethacin was given with either SKF525A or clotrimazole. These results suggest that in the STZ-induced diabetic perfused kidney, vasorelaxant metabolites derived from AA (probably COX and/or CYP450 metabolites) are increased, and may serve to regulate vascular tone.

Key words: arachidonic acid, kidney, perfusion pressure, rat, streptozotocin

Introduction

The major cause of renal failure worldwide is diabetic nephropathy, and obesity-related metabolic syndrome often contributes to the development of type 2 diabetes (Sowers and Epstein, 1995; Kambham et al., 2001). Hyperglycemia, obesity, and elevated blood pressure, along with early hyperfiltration, are major risk factors that contributing to the progression of renal damage in diabetes (Sowers and Epstein, 1995; Vivian and Rubinstein, 2002). This progression of diabetic nephropathy includes an initial increase in glomerular filtration rate (GFR), a thickening of the glomerular basement membrane, an expansion of the mesangium,
and eventually a decline in GFR (Sowers and Epstein, 1995; Vivian and Rubinstein, 2002). Moreover, renal hemodynamic changes may make an important contribution to diabetic nephropathy, and it is well known that vascular disease is a complicating feature of diabetes mellitus in humans. The reactivity of vascular smooth muscle and the endothelium to vasoactive agents in diabetes has been extensively studied in experimental animals and humans (Poston and Taylor, 1995; Kwan, 1999; De Vriese et al., 2000b; Triggle et al., 2003; Makino and Kamata, 2002; Matsumoto et al., 2004; Fitzgerald et al., 2005; Kobayashi et al., 2004, 2005). Concerning the macrovasculature, an accumulating body of evidence suggests that the relaxation responses induced in aortic strips by endothelium-dependent agents are weaker in streptozotocin (STZ)-induced diabetic rats than in non-diabetic control rats (Kamata et al., 1989, 1999, 2005; Kamata and Kobayashi, 1996; De Vriese et al., 2000; Kobayashi et al., 2000). Several reports have shown an impairment of acetylcholine (ACh)-induced endothelium-derived hyperpolarizing factor (EDHF)-signaling in the microvasculature of diabetic animals (Fukao et al., 1997; Makino et al., 2000; Matsumoto et al., 2003a,b, 2005, 2006a,c; Fitzgerald et al., 2005). Moreover, diabetes-related endothelial dysfunction has been linked to changes in eicosanoid metabolism (Sowers and Epstein, 1995; Imig, 2006).

Arachidonic acid (AA) is metabolized by the vascular endothelium and smooth muscle cells to a variety of cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P-450 (CYP450) epoxygenase products, and these metabolites exert influences over vascular tone (Sekiguchi et al., 1998; Roman et al., 2000; Roman, 2002; Fleming, 2001; Miyata and Roman, 2005; Shimamura et al., 2005; Vizoili et al., 2005; Imig, 2006). The identity and potential biological activity of some of the relevant AA metabolites remain poorly characterized (structurally and/or biologically). AA itself causes relaxation of blood vessels, an effect that is dependent on the presence of the endothelium (Pinto et al., 1987). Moreover, Pfister et al. (1996) reported that the ability of AA, but not of ACh, to induce relaxation depends on a non-COX metabolite from endothelial cells that relaxes rabbit vascular smooth muscle. In our previous studies, we found that ACh-induced endothelium-dependent relaxation was impaired in the perfused kidney of the streptozotocin (STZ)-induced diabetic rat (Kamata and Hosokawa, 1997b; Kamata and Hayashi, 1999; Kamata and Yamashita, 1999), and we provided evidence that this endothelial dysfunction may be due to decreased nitric oxide (NO) or EDHF signaling (Kamata and Hosokawa, 1997b). Moreover, we found that ACh increased the level of 6-keto-prostaglandin F_1α (a metabolite of prostacyclin) in the effluent collected from the perfused kidney of the STZ-induced diabetic rat, and we suggested that an elevated level of prostacyclin may contribute to the alteration of renal vascular tone in diabetic states (Kamata and Hosokawa, 1997b). However, the precise role played by AA metabolites in mediating the changes in renal vascular tone associated with diabetic states has yet to be established. Hence, to help clarify the effects of AA on vascular tone in the diabetic kidney, we studied the changes in perfusion pressure evoked by AA in isolated perfused kidneys from STZ-induced diabetic rats and age-matched control rats.
Methods

General

The experimental design was approved by the Hoshi University Animal Care and Use Committee, and all studies were conducted in accordance with “Guide for the Care and Use of Laboratory Animals” published by the US National Institute of Health, and “Guide for the Care and Use of Laboratory Animals” adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan).

Materials

Arachidonic acid (AA), bovine serum albumin (fraction V) (BSA), methoxamine hydrochloride, indomethacin, SKF525A, streptozotocin (STZ), and clotrimazole were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium furegrelate, SQ29548, and baicalein were from Cayman Chemical (Ann Arbor, MI, U.S.A.). Acetylcholine chloride (ACh) was from Daiichi Pharmaceuticals (Tokyo, Japan). All concentrations are expressed as the final molar concentration of the base in the organ bath.

Animal model of diabetes

Male Wistar rats (8 wk old and 200–220 g body weight) received a single injection via the tail vein of STZ 60 mg/kg dissolved in a citrate buffer. Age-matched control rats were injected with the buffer alone. Food and water were given ad libitum. The experiments described here were performed ten weeks after the injection. Ten weeks after the administration of STZ (diabetic group) or buffer (control group), plasma glucose was determined using a commercially available enzyme kit (Wako Chemical Company, Osaka, Japan), as reported previously (Kobayashi et al., 2006; Matsumoto et al., 2006b).

Preparation of the perfused kidneys

Perfusion pressure was recorded from the rat kidney as in our previous papers (Kamata and Hosokawa, 1997a, 1997b; Kamata and Mizutani, 1999). Rats were anesthetized with pentobarbital (50 mg/kg intraperitoneally) and then given an intravenous injection of 1,000 units/kg of heparin. They were killed by decapitation, the abdomen was opened by midline incision, and the left renal artery was cannulated via an incision made in the aorta. An incision was also made in the left renal vein for a cannula made from PE90 tubing. Beginning 10 min after decapitation, the kidney was perfused with warm (37°C), oxygenated (95% O₂, 5% CO₂) Krebs-Henseleit solution (KHS). This solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 25.0 NaHCO₃, 1.8 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgSO₄, 11.1 dextrose, and 0.25% BSA. A constant-flow perfusion pump (Model MP-3; Tokyo Rikakikai, Tokyo, Japan) was used for the perfusion, which proceeded at a rate of 4 ml/min through the cannula inserted into the aorta. Venous effluent was re-circulated (except during the first 60 min; see below), and mean renal perfusion pressure was 65 ± 5.8 mmHg when the renal vascular bed was perfused at the rate mentioned above. Vascular responses were detected as changes in perfusion pressure, and this was
monitored continuously with the aid of a pressure transducer (Model AP-2001; Nihon Kohden, Tokyo, Japan) and recorded on a pen recorder (Model 3021; Yokogawa, Tokyo, Japan). Following the 60-min equilibration period, the perfusion circuit was transformed into a closed system (i.e., the perfusion solution from the vein was collected in a second bath and re-circulated through the kidney) to investigate drug-induced alteration of renal perfusion pressure.

After equilibration, the perfused-kidney preparation was constricted by perfusion with $5 \times 10^{-7}$ to $3 \times 10^{-6}$ M methoxamine, which resulted in a perfusion pressure of 150–160 mmHg, and was then relaxed with $10^{-6}$ M ACh, to confirm the integrity of the endothelium. To investigate the effect of AA on perfusion, AA ($10^{-5}$ M) was added after the methoxamine ($5 \times 10^{-7}$ to $3 \times 10^{-6}$ M)-induced preconstruction had reached a plateau. To investigate the influences of indomethacin [cyclooxygenase (COX) inhibitor; $10^{-5}$ M], furegrelate [thromboxane A$_2$ (TXA$_2$)-synthase inhibitor; $10^{-5}$ M], SQ29548 (TXA$_2$-receptor antagonist; $10^{-6}$ M), baicalein [non-specific lipooxygenase (LOX) inhibitor; $5 \times 10^{-6}$ M], SKF525A [cytochrome P450 (CYP) inhibitor; $10^{-5}$ M], and clotrimazole (epoxygenase inhibitor; $10^{-5}$ M) on the AA-induced response, the perfused kidney was incubated in the appropriate solution for 20 min before the addition of methoxamine.

**Statistical analysis**

Data are expressed as the mean ± S.E.M. Multiple comparisons between treatment groups were performed using an analysis of variance (ANOVA) followed by a Bonferroni test.

**Results**

**General parameters**

At the time of the experiment, all STZ rats (non-fasted) exhibited hyperglycemia, their blood glucose concentrations ($607.8 \pm 13.4$ mg/dl, n=20) being significantly higher than those of the age-matched nondiabetic control rats (also non-fasted) ($158.2 \pm 9.7$ mg/dl, n=20; $P<0.001$). The body weight of the STZ rats ($221.3 \pm 3.1$ g, n=20) was lower than that of the control rats ($500.5 \pm 4.8$ g, n=20; $P<0.001$).

**Effects of AA on perfusion pressure in methoxamine-preconstricted kidneys**

In perfused methoxamine-preconstricted kidneys from age-matched control rats and STZ-induced diabetic rats, AA ($10^{-5}$ M) elicited a transient contraction (i.e., an increase in perfusion pressure), and then a sustained relaxation (i.e., perfusion pressure declined towards, or below, the methoxamine-induced plateau level) (Figs. 1 and 2). The maximum amplitude of the contractile response was significantly smaller in STZ-induced diabetic rats than in age-matched control rats. On the other hand, that of the relaxation response was significantly greater in STZ-induced diabetic rats than in the age-matched controls (Fig. 2).

**Indomethacin**

Indomethacin ($10^{-5}$ M) did not affect the AA-induced contractile response in either the age-matched control or STZ-induced diabetic group (Fig. 3). Interestingly, in the STZ-induced
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Indomethacin significantly decreased the AA-induced relaxation component (Fig. 3).

**Furegrelate and SQ29548**

To assess the influence of TXA₂ on the AA-induced changes in perfusion pressure, we tested the effects of furegrelate (TXA₂-synthase inhibitor; 10⁻⁵ M) or SQ29548 (TXA₂-receptor antagonist; 10⁻⁶ M). Neither furegrelate (Fig. 4) nor SQ29548 (Fig. 5) had any significant effects on the AA-induced contraction and relaxation responses in either age-matched controls or STZ-induced diabetic rats.
To assess the influence of the LOX pathway on the AA-induced response, we tested the effect of baicalein (non-specific LOX inhibitor; 5 × 10⁻⁶ M). Baicalein (Fig. 6) had no significant effects on the AA-induced contraction and relaxation responses in either age-matched controls or STZ-induced diabetic rats.

**Baicalein**

To assess the influence of the LOX pathway on the AA-induced response, we tested the effect of baicalein (non-specific LOX inhibitor; 5 × 10⁻⁶ M). Baicalein (Fig. 6) had no significant effects on the AA-induced contraction and relaxation responses in either age-matched controls or STZ-induced diabetic rats.

**SKF525A and clotrimazole**

To assess the influence of CYP450 enzymes on the AA-induced response, we tested the effects of SKF525A (CYP450 inhibitor; 10⁻⁵ M) and clotrimazole (nonselective epoxygenase
In the STZ-induced diabetic kidney, but not in the controls, SKF525A significantly decreased both the contraction and relaxation components of the AA-induced response (Fig. 7). Clotrimazole did not affect the AA-induced contractile response in either the age-matched control or STZ-induced diabetic group (Fig. 8). However, in the STZ-induced diabetic kidney, clotrimazole significantly decreased the AA-induced relaxation component (Fig. 8).
SKF525A and clotrimazole with indomethacin

To look for possible additive effects of COX and CYP450 enzymes on the AA-induced relaxation response in the perfused diabetic kidney, we evaluated the effect of SKF525A (10⁻⁵ M) or clotrimazole (10⁻⁵ M) in the presence of indomethacin (10⁻⁵ M). When the plateau perfusion pressure during the AA-induced sustained relaxation in the diabetic perfused kidney was defined as baseline, each agent given alone (indomethacin, SKF525A, or clotrimazole) induced a significant increase in perfusion pressure (Fig. 9). Moreover, in the presence of indomethacin, both SKF525A and clotrimazole further increased perfusion pressure (Fig. 9).

**Fig. 4.** Effects of furegrelate (10⁻⁵ M) on the responsiveness to arachidonic acid (AA; 10⁻⁵ M) shown by perfused kidneys preconstricted with methoxamine (5 × 10⁻⁷ to 3 × 10⁻⁶ M). (A) Time-course data and (B) maximum (I) and minimum (II) amplitude (measured as shown in Fig. 1) in the response to AA in the perfused kidneys of age-matched control and STZ-induced diabetic rats. Each data-point represents the mean ± S.E.M. from 8 preparations; the S.E.M. is included only when it exceeds the dimension of the symbol used. **,** P<0.01; ***,** P<0.001 vs. age-matched control group.
The principal findings made in the present study, on rats, were: 1) AA elicited a transient contraction followed by a sustained relaxation in the methoxamine-preconstricted perfused kidney, 2) the amplitude of the AA-induced contraction was smaller in the STZ-induced diabetic kidney than in the age-matched controls, and 3) the amplitude of the AA-induced relaxation was greater in the diabetic group, an enhancement that might be due to increased COX and/or CYP450 metabolites.

Discussion

The principal findings made in the present study, on rats, were: 1) AA elicited a transient contraction followed by a sustained relaxation in the methoxamine-preconstricted perfused kidney, 2) the amplitude of the AA-induced contraction was smaller in the STZ-induced diabetic kidney than in the age-matched controls, and 3) the amplitude of the AA-induced relaxation was greater in the diabetic group, an enhancement that might be due to increased COX and/or CYP450 metabolites.
AA metabolites are vital for the proper regulation of renal hemodynamics and, when not adequately controlled, can contribute to renal vascular injury and end-stage renal disease (Imig, 2006). Three major enzymatic pathways—the COX, CYP450, and LOX pathway—are responsible for the metabolism of AA metabolites to bioactive eicosanoids. These eicosanoids can dilate or constrict the renal vasculature, and serve to maintain vascular resistance in the face of changing level of vasoactive hormones. In the present study, we investigated the effects of inhibitors of these enzymes (i.e., COX, CYP450, and LOX) on the responses induced by AA in methoxamine-preconstricted perfused kidneys from diabetic rats.
Within the vascular structures of the kidney, COX enzymes are responsible for the conversion of AA into PGG/PGH₂ (Imig, 2000). The kidney constitutively expresses both COX-1 and COX-2 isoforms, and each of these isoforms contributes to the regulation of vascular function (Imig, 2000; Cheng and Harris, 2004). The second step in the enzymatic reactions occurs through the actions of PG synthase and TX synthase, which convert PGH₂ into biologically active metabolites. In the present study, although the AA-induced contractile response in perfused kidneys was not altered by the presence of the COX1/COX2 inhibitor indomethacin in either the controls or the diabetics, the enhanced AA-induced relaxation seen...
in the perfused kidneys of the STZ-induced diabetic group was greatly inhibited by indomethacin (Fig. 3). On the other hand, incubation with the TXA$_2$-synthase inhibitor furegrelate (Fig. 4) or with the TXA$_2$-receptor antagonist SQ29548 (Fig. 5) did not alter the AA-induced contractile or relaxation responses in perfused kidneys from either group. These results suggest that COX-derived vasodilator metabolites alter renal perfusion pressure in diabetic conditions (at least, in the STZ-diabetic condition). In fact, several studies have linked diabetes with enhanced COX-2 generation of prostanoids in the kidney (Komers et al., 2001; Imig, 2006). Moreover, several reports have suggested that enhanced production of vasodilator
PGs by the glomeruli may be only associated with an increased GFR in STZ-induced diabetes, but also implicated in the progression of diabetic nephropathy (Cheng et al., 2002; Cheng and Harris, 2004; Natarajan and Nadler, 2004). Furthermore, in a previous report, we noted an ACh-induced increment in 6-keto-prostaglandin F\(_1\alpha\) (a metabolite of prostacyclin) in the perfusate issuing from the STZ-induced diabetic kidney (Kamata and Hosokawa, 1997b).

LOX enzymes metabolize AA to generate leukotrienes, hydroxyeicosatetraenoic acid (HETEs) and lipoxins, and these LOX metabolites have inflammatory actions, affect smooth muscle cell growth, and regulate renal hemodynamics (Imig, 2006). In the present study, incubation with the non-specific LOX inhibitor baicalein (Fig. 6) failed to modify the AA-induced contractile or relaxation responses in either group. These results suggest that LOX-derived metabolites play little or no role in the modulation of the vascular response of the kidney to AA.

CYP450 enzymes are expressed within vascular and tubular structures in the kidney, and their actions have a variety of functional consequences, depending on the AA product [e.g., EET

**Fig. 9.** Effects of indomethacin (10\(^{-5}\) M), SKF525A (10\(^{-5}\) M) and clotrimazole (10\(^{-5}\) M) on the arachidonic acid (AA; 10\(^{-5}\) M)-induced relaxation component (shown in Fig. 1 as II) in the perfused, methoxamine (5 \times 10^{-7} to 3 \times 10^{-6} M)-preconstricted kidney of the STZ-induced diabetic rat. Ordinate shows increase in perfusion pressure (mmHg) (0% being defined as the stable level of the AA-induced relaxation in the non-treatment group). Each data-point represents the mean ± S.E.M. from 8 preparations; the S.E.M. is included only when it exceeds the dimension of the symbol used. **, P<0.01; ***, P<0.001 vs. non-treatment group. ##, P<0.01; ###, P<0.001 vs. indomethacin-treated group. ††, P<0.01 vs. clotrimazole-treated group.
(epoxygenase metabolite) is a vasodilator, whereas 20-HETE (hydroxylase metabolite) is a vasoconstrictor] (Imig, 2000, 2006; Miyata and Roman, 2005). In the present study, although the AA-induced contractile and relaxant responses in the control group were not altered in the presence of the CYP450 inhibitor SKF525A, both the AA-induced contraction and enhanced relaxation in the STZ-induced diabetic group were greatly inhibited by SKF525A (Fig. 7). Although SKF525A reportedly inhibits has not only CYP450 activity but also Ca\(^{2+}\) influx (Bruce and Elliott, 2000), we suggest that the effect of SKF525A on renal perfusion pressure we observed in our diabetic group resulted from its inhibitory action on CYP450 activity because the concentration of SKF525A we used did not affect perfusion pressure in the control kidney. This interpretation is supported by our finding that clotrimazole (an other epoxygenase inhibitor) inhibited the enhanced AA-induced relaxation seen in the STZ-induced diabetic group (Fig. 8). The above results suggest that CYP450-derived vasodilator metabolites modify the perfusion pressure in the perfused kidney under STZ-diabetic conditions. Furthermore, when the plateau level of perfusion pressure achieved during the AA-induced sustained relaxation in the diabetic perfused kidney was defined as baseline, an additional increase in perfusion pressure was seen when either SKF525A or clotrimazole was given with indomethacin (versus the increase seen with indomethacin alone) (Fig. 9). Thus, these results suggest the existence of a synergistic interaction was in the diabetic kidney between COX and CYP450 metabolites. This speculation is in line with the previous findings (a) that COX can convert 20-HETE into the renal vasodilator 20-OH-PGE\(_2\) and constrictor 20-OH-PGH\(_2\) (McGiff and Quilley, 1999) and (b) that 5,6-EET, a renal vasodilator metabolite produced from AA via the action of CYP450, requires COX for the expression of its vasoactivity (since the response is inhibited by indomethacin and other aspirin-like drugs) (Carroll et al., 1993).

We found previously that in the perfused kidney, AA (10\(^{-5}\) M) did not, by itself, induces vasoconstriction (Kamata and Hosokawa, 1997b), but did transiently increase the perfusion pressure in the methoxamine-preconstricted preparation. AA is known to be a Ca\(^{2+}\)-sensitizing agent (Somlyo and Somlyo, 1994; Hirano et al., 2004). It increases the phosphorylation of myosin light chain 20 (MLC20) and causes contraction of smooth muscle at a constant Ca\(^{2+}\) by inhibiting myosin light-chain dephosphorylation (Gong et al., 1992). Moreover, AA elicits this Ca\(^{2+}\)-sensitization of smooth muscle contraction through its activation of Rho-kinase (Araki et al., 2001). In the present study, the AA-induced contractile effect was weaker in the diabetic group than in the control group, and was significantly inhibited by SKF525A treatment only in the former group. Possibly, this reduced contractile effect of AA in the diabetic perfused kidney may be attributable not only to an impairment of the responsiveness of the contractile machinery in smooth muscle cells to AA, but also an imbalance among the products of CYP450 metabolism. However, further studies will be required on this point.

In conclusion, the present results suggest that in the perfused kidney of the STZ-induced diabetic rats, vasorelaxant metabolites of AA (probably COX and CYP450 metabolites) are increased, and may serve to regulate vascular tone. Such alterations on AA metabolism may contribute to the development and progression of diabetic nephropathy.
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