Diabetes Undermines Estrogen Control of Inducible Nitric Oxide Synthase Function in Rat Aortic Smooth Muscle Cells Through Overexpression of Estrogen Receptor-β

Adriana Maggi, PhD*; Andrea Cignarella, PhD*; Alessia Brusadelli, PhD; Chiara Bolego, PhD; Christian Pinna, PhD; Lina Puglisi, PhD

Background—Previous reports from our group have shown that 17β-estradiol reduces the synthesis and activity of inducible nitric oxide synthase (iNOS) in rat aortic smooth muscle cells (SMC) in response to inflammatory mediators. In this study, we investigated the effect of 17β-estradiol on iNOS function in aortic SMC from streptozotocin-diabetic rats.

Methods and Results—Comparative analysis of NO release and of iNOS mRNA and protein content after 24-hour stimulation with a cytokine mixture revealed milder iNOS activation in diabetic than in control SMC. Furthermore, 17β-estradiol dose-dependently blocked iNOS synthesis and activity in control but not in diabetic SMC. The defective estrogen response in diabetic SMC at 24 hours could not be attributed to reduced expression of estrogen receptors (ER). In fact, mRNA and protein levels of ERα and, to a greater extent, of ERβ, were increased in diabetic compared with nondiabetic SMC. Cytokines decreased ERα and ERβ expression in both groups. However, 17β-estradiol dose-dependently restored the expression of ERα but further downregulated that of ERβ, indicating a differential regulation of ER isoforms.

Conclusions—Estrogenic control of iNOS was impaired in diabetic SMC. This was associated with a larger increase of ERβ than of ERα protein, whereas 17β-estradiol regulated the two isoforms in an opposite fashion. Thus, modifications in the estrogen modulation of iNOS and in the expression pattern of ER may be involved in diabetic vascular dysfunction. (Circulation. 2003;108:211-217.)

Key Words: myocytes ■ nitric oxide synthase ■ diabetes mellitus ■ hormones

Diabetes is a cardiovascular disease.1 Substantial evidence indicates that diabetic vascular dysfunction is associated with marked alterations of nitric oxide (NO) pathways.2 In the presence of high blood glucose levels, the increased vascular generation of oxygen-derived free radicals, primarily superoxide anions, quenches the biological activity of NO.2–4 This may result in a transient increase in NO synthesis through upregulation of endothelial NO synthase (eNOS) in endothelial cells, and even more so through activation of the inducible NOS (iNOS) isoform in smooth muscle cells (SMC) and macrophages. The sustained accumulation of high NO levels generated by iNOS can be toxic through reaction with superoxide to yield peroxynitrite, thereby playing a central role in the pathophysiology of inflammation and oxidant stress.5 Thus, the iNOS-mediated increase in NO formation may be a major mediator of diabetic vascular dysfunction. Accordingly, functional expression of iNOS has been reported in SMC from the superior mesenteric arteries of rats with chronic diabetes.6

We previously showed that diabetes impairs vascular function in female streptozotocin-diabetic rats by interfering with NO pathways.7 In women, diabetes abolishes the gender advantage in cardiovascular risk typical of premenopause.8 This protection is thought to be mediated, at least in part, by endogenous 17β-estradiol (E2) through mechanisms still under investigation.

Thus far, two estrogen receptors (ER) have been identified: ERα and ERβ. These proteins are encoded by two separate genes, are distinct structurally and functionally, and are expressed in endothelial cells,9 SMC,10,11 and macrophages.12 In endothelial cells, E2 increases the expression of eNOS, thereby controlling the tone of underlying SMC,13 and may also rapidly activate eNOS through ERα-mediated mechanisms not involving gene expression.14,15 Conversely, E2 blocks the synthesis of iNOS induced by inflammatory stimuli in SMC11 and macrophages,12 suggesting that the beneficial effect of the hormone arises from a combined action on different vascular cell types.
The aim of the present study was to further investigate the effects of E₂ on the expression and activity of iNOS in cultured aortic SMC from diabetic rats. In this study, we show that iNOS response to inflammatory events in SMC from diabetic rats is less sensitive to estrogen, probably on account of altered ERα/ERβ ratio. These findings indicate possible mechanisms underlying the increased risk of cardiovascular disease in diabetic premenopausal women.

Methods

Materials
E₂, L-arginine, sepiapterin, ascorbic acid, l-nitroarginine-methyl ester (L-NAME), S-ethylisothiourea (EIT), and streptozotocin (STZ) were obtained from Sigma. The anti-rabbit iNOS polyclonal antibody was purchased from Transduction Laboratories (Lexington, Ky). The anti-ERα and anti-ERβ antibodies were kindly provided by Dr Geoffrey L. Greene (University of Chicago). Oligodeoxynucleotides were synthesized by Amersham Biosciences.

Diabetes Induction
Diabetes was induced by intravenous injection of STZ (65 mg/kg) in male Sprague-Dawley rats weighing 200 to 225 g (Charles River, Calco, Italy). STZ was freshly dissolved in citrate buffer (pH 4.5); control animals were injected with vehicle. Diabetes induction was considered successful when glycosuria was detectable 1 week after treatment. When the animals were killed, they had plasma glucose levels >25 mmol/L. The procedures followed were in accordance with institutional guidelines of the University of Milan.

Cell Culture
SMC were obtained from aortic intimal-medial layers of nondiabetic and 28-day diabetic rats according to Ross. Cells were grown in medium 199 (M199) as previously described. The medium was replaced with phenol red–free M199 with 10% FCS for 5 days whenever the effects of E₂ were tested. Twenty-four hours before replacing the medium, the SMC were synchronized in medium containing 0.4% FCS and stimulated with cytokine mixture comprising 10 ng/mL IL-1β, 10 ng/mL IFN-γ, 25 ng/mL TNF-α, and 10 μg/mL LPS for 24 hours. Increasing concentrations of E₂ (10⁻¹¹ to 10⁻⁹ mol/L) were added at the same time as cytokines where indicated. Results are mean±SEM of 6 to 7 independent experiments performed in duplicate. *P<0.01 vs untreated; **P<0.05 vs untreated diabetic and cytokine-treated control; §§P<0.01 vs cytokine-treated control; §§§P<0.05 vs cytokine-treated diabetic (ANOVA).

Gene Expression Analysis
Total RNA was extracted from SMC (2×10⁵ cells/well) and rat uterus (as a positive control for ER mRNA expression) with the Bio/RNA-X Cell kit (Bio/Gene, Kimbolton, UK). RNA was reverse transcribed using MMLV reverse transcriptase H(-) (Promega). Amplification of ERα and ERβ cDNAs was carried out with the use of published primer sequences. Amplification of iNOS cDNA was achieved by using the primer pair 5'-ATGCGCCGACCTGTGCTTGC-3' (reverse) and 5'-TTGACCAGGAGCCACGAG-3' (forward). The intensity of PCR product bands was normalized to that of the housekeeping gene GADPH by densitometric analysis with the use of the Quantity-One software (Bio-Rad).

Western Blot Analysis
After quantification by Lowry’s method, 28 μg of cell protein was loaded onto discontinuous gradient SDS-PAGE (10% to 5%) gels. After electrophoresis, proteins were transferred to nitrocellulose membrane and incubated first with the primary antibodies (all 1:10000) overnight, then with the peroxidase-conjugated secondary antibody (Bio-Rad) for 1 hour. Proteins were detected by chemiluminescence (Amersham Biosciences).

Figure 1. Effects of E₂ on cytokine-induced nitrite accumulation in culture medium of aortic SMC from control and diabetic rats. SMC were grown in phenol red–free M199 medium for 5 days, synchronized in 0.4% FCS for 24 hours, and stimulated with cytokine cocktail (10 ng/mL IL-1β, 10 ng/mL IFN-γ, 25 ng/mL TNF-α, and 10 μg/mL LPS) for 24 hours. Increasing concentrations of E₂ (10⁻¹¹ to 10⁻⁹ mol/L) were added at the same time as cytokines where indicated. Results are mean±SEM of 6 to 7 independent experiments performed in duplicate. *P<0.01 vs untreated; **P<0.05 vs untreated diabetic and cytokine-treated control; §§P<0.01 vs cytokine-treated control; §§§P<0.05 vs cytokine-treated diabetic (ANOVA).

Statistical Analysis
Data were obtained from at least 3 independent experiments, each value representing mean±SEM of duplicate or triplicate determinations. Differences between mean values of multiple groups were analyzed by 1-way ANOVA with Fisher’s analysis. Values of P<0.05 were considered statistically significant.

Results

E₂ Prevents Cytokine-Induced Secretion of Nitrite in SMC From Control But Not From Diabetic Rats
In cultured control SMC exposed to a cytokine mixture for 24 hours, nitrite levels in the medium increased ~4.5-fold (Figure 1), but this was significantly opposed by E₂ (10⁻¹¹ to 10⁻⁹ mol/L). Basal nitrite levels were not affected by E₂ in either group. In SMC from diabetic rats, cytokines increased nitrite production only 2.4-fold, and E₂ did not block cytokine effects but led to a slight dose-dependent increase in nitrite levels (Figure 1). This suggests that diabetes induced alterations in SMC metabolism that persisted in the absence of high glucose levels and affected the estrogenic control of nitrite production.

Some vascular dysfunction can be reversed by increasing NOS substrate or adding antioxidants. We then evaluated whether the altered response to cytokine stimulation in diabetic SMC was related to shortage of iNOS substrate or cofactors or to increased oxidant activities. As shown in the Table, addition of increasing concentrations of the iNOS
substrate L-arginine or cofactor sepiapterin did not augment nitrite accumulation in the medium of diabetic SMC, suggesting that those were not limiting steps for iNOS activity. Similarly, addition of either ascorbic acid or lercanidipine (a vascular-selective calcium channel blocker with marked antioxidant properties) failed to affect nitrite production, suggesting that no increased oxidant burden impaired iNOS activity.

In both SMC groups, the cytokine-induced nitrite increase was reversed by the nonselective NOS inhibitor L-NAME and the relatively selective iNOS inhibitor EIT (Figure 2), suggesting that it was secondary to iNOS induction. After 24-hour cytokine challenge, nitrite levels were again greater in the media of control SMC than in those of diabetic SMC. However, at 48 hours, nitrite content in the two groups was comparable. Further investigation showed that such differences reflected a distinct temporal pattern of iNOS protein induction. In fact, we consistently measured the highest iNOS protein levels after 48-hour cytokine treatment in diabetic SMC and after 24 hours in control SMC (Figure 3). After 72 hours, iNOS protein levels decreased, yet were significantly higher than in untreated cells. This delayed response to cytokine stimulation in diabetes was not restricted to isolated SMC but was also observed in whole aortic rings in culture under identical conditions (not shown).

### E2 Exerts Opposite Modulation of iNOS Synthesis in Control and Diabetic SMC

We then evaluated the content of iNOS mRNA and protein after E2 treatment. As expected, iNOS mRNA was undetectable in untreated SMC from either group, as shown by semiquantitative RT-PCR. In control SMC, cytokine stimulation for 24 hours increased iNOS mRNA content; a modest induction was observed in diabetic SMC as well. However, the overall effect of E2 was opposite in the two groups. In fact, E2 (10⁻¹¹ to 10⁻⁹ mol/L) dose-dependently reduced iNOS mRNA levels in control SMC but increased iNOS mRNA in diabetic SMC severalfold compared with cytokine-treated diabetic and ≈2-fold compared with cytokine-treated control SMC (data not shown).

Western blot experiments showed that E2 reduced in a dose-dependent manner iNOS protein content in cytokine-treated control SMC (Figure 4A). By contrast, treatment with E2 did not affect iNOS protein levels in diabetic SMC despite increased mRNA levels. As shown in Figure 4B, after 48-hour cytokine stimulation, E2 was about as effective in control as in diabetic SMC at reducing iNOS protein content, indicating that the early difference in response to E2 between control and diabetic SMC leveled off at later time points. Similar results were obtained for iNOS activity (data not shown).

### Effects of Different Supplementations on Nitrite Content in Medium of Aortic SMC From Diabetic Rats

<table>
<thead>
<tr>
<th>Treatment/Concentration, μmol/L</th>
<th>Nitrite, μmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.041±0.014</td>
</tr>
<tr>
<td>Cytokines</td>
<td>0.065±0.011*</td>
</tr>
<tr>
<td>Cytokines + L-arginine 50</td>
<td>0.067±0.009</td>
</tr>
<tr>
<td>Cytokines + L-arginine 100</td>
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<tr>
<td>Cytokines + L-arginine 500</td>
<td>0.073±0.010</td>
</tr>
<tr>
<td>Cytokines + ascorbic acid 200</td>
<td>0.077±0.013</td>
</tr>
<tr>
<td>Cytokines + ascorbic acid 500</td>
<td>0.064±0.016</td>
</tr>
<tr>
<td>Cytokines + ascorbic acid 2000</td>
<td>0.079±0.008</td>
</tr>
<tr>
<td>Cytokines + lercanidipine 0.1</td>
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<tr>
<td>Cytokines + sepiapterin 30</td>
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<td>Cytokines + sepiapterin 100</td>
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</tr>
<tr>
<td>Cytokines + sepiapterin 300</td>
<td>0.073±0.006</td>
</tr>
</tbody>
</table>

SMC were incubated with cytokines for 24 hours in the presence of increasing concentrations of the indicated compounds. At the end of incubation, medium aliquots were assayed for nitrite content by the Griess’ reaction (see Methods section). Data are expressed as mean±SEM of 3 to 6 independent experiments in duplicate. *P<0.05 vs control.

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Figure 2. Nitrite production by control and diabetic SMC after 24-hour and 48-hour incubation with cytokines. SMC were grown in M199 medium+10% FCS until confluence, synchronized in 0.4% FCS for 24 hours, and stimulated as described in the legend to Figure 1 for the indicated time in the presence of the NOS inhibitors L-NAME (0.3 mmol/L) and EIT (10 μmol/L). Nitrite production was measured in medium aliquots by Griess’ reaction. Data are expressed as mean±SEM of 5 to 7 independent experiments, each performed in duplicate. *P<0.01 vs basal; §P<0.01 vs cytokines.
this effect was associated with a significant change in ERα and ERβ relative content. These differences may be relevant to diabetic vascular dysfunction.

Data from our group and other groups indicate that the E2 antagonism to the synthesis of inflammatory proteins is mediated by ER because it occurs in cell cultures at hormone concentrations compatible with ER transcriptional activation, is blocked by specific ER antagonists, and is linked to ER expression.11,12,23,24 In vitro transfection experiments show that ER is indispensable to E2 regulation of iNOS transcription. There is little evidence, however, for a direct interaction of estrogen with the iNOS promoter,25 which lacks canonical estrogen responsive elements. As proposed for the effect of estrogens on other molecules of the inflammatory cascade, it is conceivable that iNOS mRNA synthesis is controlled by ERs by interaction with transcription factors such as NF-κB, AP-1, or STATs.26,27 Paech et al28 reported that E2 has opposite influence on NF-κB and AP-1 transcription activity, depending on its association with ERα or ERβ. Considering a differential effect of the two ER subtypes on iNOS expression, we propose that the altered ERα/ERβ ratio reported here for diabetic SMC, and in particular the remarkable upregulation of ERβ, underlies the loss of E2 negative control on iNOS activity. ERα and ERβ expression was increased in diabetic compared with control SMC, with a relative increase in ERα and ERβ protein levels of ~1.4- and 2.5-fold, respectively. Although no experiments were performed in this study to distinguish functionally between ERα and ERβ, our findings suggest that the increased ERβ expression was associated with the defective response to E2 and to cytokines observed in diabetes. Supporting this hypothesis are recent studies in ERβ-deficient mice describing an important role for ERβ in regulating vascular function and blood pressure.29 The authors showed that ERβ is involved in estrogen-induced accumulation of iNOS protein in endothelium-denuded aortic rings as well as iNOS gene transcription in normal vessels. This lends further support to the view that the altered estrogenic control of iNOS function in diabetic SMC was mediated mainly by ERβ.

The observation that ERα and ERβ protein content was regulated by E2 in an opposite fashion in aortic SMC (Figures 5 and 6) provides further evidence for the potential divergent effects of the two receptors.28,30 By upregulating ERα, E2 may enhance the protective antiinflammatory effects mediated by this receptor subtype in these cells while limiting potential proinflammatory effects with ERβ downregulation. Interestingly, enhanced transcription of both ER subtypes occurred in diabetic cells, although the increase in protein level was more evident for ERβ. It is conceivable that ER protein underwent translational regulation, as was the case for iNOS; this phenomenon has been reported to occur in diabetes.31,32 Accordingly, the cytokine-dependent synthesis of iNOS was delayed and significantly blunted in diabetic cells possibly due to altered levels of transcription factors or of intracellular signaling molecules such as intracellular Ca²⁺, as suggested by previous studies in SMC.33 The cytokine response itself was blunted but not abolished in diabetic SMC at the 24-hour time point. Therefore, the altered time course of iNOS response to cytokines and the corresponding loss of estro-
genic effect on this phenomenon, along with the changes in the ER expression pattern, appeared to be part of diabetic vascular dysfunction in aortic SMC.

Activation of iNOS in SMC leads to rapid production of high amounts of NO, which causes pathological vasodilation. In addition, excess NO may interact with superoxide to generate peroxynitrite, which in turn modifies the function of several proteins, contributing to the tissue damage occurring in vascular sepsis and inflammation.18 Our results may partially explain why diabetes abolishes the gender-specific

Figure 4. Effects of E2 (10^{-11} to 10^{-9} \text{mol/L}) on cytokine-induced iNOS protein synthesis in control and diabetic SMC. SMC were grown and incubated for 24 hours (A) and 48 hours (B), as described in the legend to Figure 1. Amount of iNOS in cell lysates was quantified by scanning densitometry. Representative Western blots for iNOS are shown. Data are expressed as mean±SEM of 3 to 5 independent experiments. \(*P<0.01\) vs cytokines (ANOVA).

Figure 5. Effects of E2 (10^{-11} to 10^{-9} \text{mol/L}) on mRNA levels of ER in control and diabetic SMC. SMC were grown and stimulated as described in the legend to Figure 1. Total RNA was extracted and cDNAs of ER\(\alpha\) and ER\(\beta\) amplified as detailed in the Methods section. Levels of mRNA for ERs were normalized to GAPDH mRNA by densitometry analysis. In control SMC, cytokine treatment with or without E2 led to virtually undetectable ER\(\beta\) mRNA levels. Representative RT-PCR assays are shown. Data are expressed as mean of 3 independent experiments.
vascular protection afforded by estrogen reported in epidemiological studies. Thus, understanding the mechanism for the vascular alterations in estrogen response and ER levels in diabetes would be particularly relevant from a clinical perspective. Estrogen inhibits the iNOS activation associated with ovariectomy, uraemia, transplantation, and inflammatory stimuli. Estrogen also appears to be effective in clinical conditions sharing inflammatory features such as multiple sclerosis, Alzheimer’s disease, osteoporosis, and periodontitis, indicating that the beneficial effects of estrogen reflect its antiinflammatory action. In view of the possible role of ERβ in the development of diabetic vascular dysfunction, it would be interesting to assess the role of ERβ in other inflammatory disorders in which the hormone exerts protective effects. These studies might eventually lead to the development of novel drugs for prevention and treatment of inflammatory disease affecting the aging woman.

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