Chapter 6

TGF-β antibody treatment restores impaired aortic contraction to angiotensin II in streptozotocin-induced diabetic rats

Submitted
Abstract
Impaired vasoconstriction to angiotensin II (AII) in diabetes may be related to impaired calcium mobilization via the inositol 1,4,5-trisphophate receptors (InsP3R). As TGF-β downregulates the InsP3Rs in cell culture we determined whether anti-TGF-β antibody (anti-T) treatment in diabetic rats reverses the effects of diabetes on aortic contractions to AII. Three groups (n=6 per group) were evaluated at week 2: rats made diabetic with streptozotocin, anti-T treated diabetic rats, and control rats. Blood glucose and body weights were similar in both diabetic groups. In diabetic rats, maximal isometric aortic contractions to AII were decreased by 50% (p<0.05 vs. control), without a significant change in EC50. Treatment of diabetic animals with anti-T completely restored AII mediated contractions. Semi-quantitative RT-PCR was performed to evaluate levels of the InsP3R isoforms and AT1R mRNA in the aortic samples. InsP3R-1 and InsP3R-3 mRNA levels were decreased in diabetic animals (~25% and -38%, respectively, p<0.05 vs. control). Treatment with anti-T normalized InsP3R-1 mRNA levels and partially restored InsP3R-3 mRNA levels. AT1R mRNA levels increased in the diabetic group and increased further with antibody treatment.
In conclusion, impaired aortic contractions to AII in diabetes is associated with a decrease in InsP3R-1 and InsP3R-3 mRNA levels and an increase in AT1R levels. Anti-T treatment restored InsP3R-1 levels and AII-induced aortic contraction. We suggest that hyperglycemia in diabetes attenuates AII response through TGF-β mediated down regulation of InsP3R-1.
Introduction:

Vascular dysfunction is a common complication of diabetes. In early and uncomplicated stages of diabetes, reduced peripheral resistance (Mathiesen et al., 1985; Thuesen et al., 1988) and increased blood flow in retina (Kohner et al., 1975), kidney (Mogensen, 1972) and skin (Houben et al., 1992) have been described. Enhanced blood flow may be explained by an increase in the production or effect of vasodilatory mediators such as nitric oxide (Graier et al., 1993) and/or by a decrease in the production or sensitivity to vasoconstrictive agents. With respect to macrovessel function, impaired responses to vasoconstrictors such as angiotensin II (AII) (Cavaliere and Taylor, 1981; Beenen et al., 1996; Turlapaty et al., 1980) and endothelin (Utkan et al., 1998) has been observed in aortas of diabetic rats. This would suggest that sensitivity to vasoconstrictors contribute to the vasodilation of early diabetes.

The vasoconstrictive properties of AII are mediated through the AT1R, a G-protein coupled seven transmembrane spanning receptor (Furuta et al., 1992). Stimulation of AT1R results in the formation of diacylglycerol and inositol 1,4,5-trisphosphate (InsP₃)(Griendling et al., 1988). InsP₃ binds to intracellular receptors (InsP₃R) situated on internal calcium stores and thus releases calcium into the cytosol (Berridge, 1993). In smooth muscle cells this calcium release results in contraction (Griendling et al., 1988). Cloning studies have shown that three types (InsP₃R-1, -2 and -3) are expressed in both rat and human tissue (Newton et al., 1994; Miyawaki et al., 1990; Yamamoto et al., 1994). Although various tissues exhibit discrete distribution of the various InsP₃R isoforms, little is known about their functional differences. We have recently found that kidneys from diabetic rats exhibit decreased expression of the type I InsP₃R isoform (Sharma et al., 1999), therefore we speculate that the impaired AII mediated vasoconstriction in diabetic animals may be caused by downregulation of InsP₃Rs.

Several observations suggest a role for Transforming Growth factor (TGF)-β in mediating diabetic vascular dysfunction. Up-regulation of TGF-β has been reported in mouse and rat models of diabetic kidney disease (Sharma et al., 1999) and in high glucose treated cultured mesangial cells (Hoffman et al., 1998). In a murine model of diabetic kidney disease, administration of neutralizing anti-TGF-β antibodies (anti-T) resulted in prevention of glomerular hypertrophy (Sharma et al., 1996). As diabetic glomerular hypertrophy is associated with increased glomerular blood flow it is possible that TGF-β may have effects on vascular regulation. In support of a possible
role for TGF-β to regulate vascular function in diabetes, prior studies have established that TGF-β has an important role in regulating NO production (Perrella et al., 1996a; Perrella et al., 1994; Junquero et al., 1992) and TGF-β receptors have been found to be increased in the aortic walls from diabetic patients (Kanzaki et al., 1997).

One of the observed effects of TGF-β is down-regulation of InsP₃R-1 in mouse mesangial cells, smooth muscle-like cells of the glomerulus (Sharma et al., 1997), which is associated with impaired InsP₃-mediated calcium release (manuscript under review). We have also observed down-regulation of InsP₃R-1 and InsP₃R-3 by TGF-β in rat renal vascular smooth muscle cells (unpublished data). Based on the above we hypothesize that the impaired aortic contraction to AII in diabetic rats is caused by TGF-β mediated downregulation of InsP₃Rs. Therefore in the present study we determined the effects of anti-T treatment on aortic contractions to AII in streptozotocin–induced diabetic rats. In addition, we investigated mRNA levels of the three isoforms of the InsP₃R and AII receptor type I (AT1R) in isolated aortic rings.

Materials and Methods:

Animals
Sprague-Dawley rats (n=12) weighing 220-260 g were made diabetic by a single intraperitoneal injection of STZ (65 mg/kg body wt) in 10 mmol/l sodium citrate, pH 5.5. Controls were injected with buffer alone (n=6). The levels of blood glucose were determined 2 days after injection and rats with blood glucose >16 mmol/l were used as diabetic rats. The diabetic rats were treated with 1-2 units of Humulin (70/30 preparation) subcutaneously each day to maintain glycemic levels between 300-500 mg/dl and avoid marked wasting. The diabetic rats were divided into two groups of 6 with one group receiving vehicle and the other group receiving anti-T. anti-T was administered intraperitoneally at a dose of 3 mg on alternate days over a 14 day period. Diabetic and nondiabetic rats were given standard rodent diet and water ad libitum. Animals were housed separately and maintained in a temperature- and light-controlled environment. All protocols in this study were approved by the Institutional Animal Care and Use Committee.

Antibody generation
Neutralizing anti-TGF-β antibody was harvested from ascitic fluid of mice injected with the 2G7 hybridoma cell line (kindly provided by Genentech,
South San Francisco, Ca$^{2+}$), and purified by affinity chromatography on Protein A columns. Reactivity of the antibody was evaluated using a mink lung bioassay (Sharma et al., 1996). The bioassay employing mink lung cells that were stably transfected with the TGF-β responsive PAI-1 promoter linked to a luciferase reporter gene was kindly provided by Dr. Daniel Rifkin (New York University) (Abe et al., 1994). Antibody preparations were sterile filtered and administered in buffered saline.

**Harvesting of tissue**

Rats were killed by cervical dislocation. After excision, rat thoracic aortas were cut into rings and randomly used for either contraction measurements or mRNA extraction. Rings to be used for aortic contraction measurements were transferred to oxygenated Krebs medium, while the rings for mRNA isolation were immediately frozen in liquid nitrogen and stored at -85°C.

**Aortic ring preparation and isometric contraction measurement**

Aortic rings of approximately 3 mm wide were mounted in a 10 ml organ bath containing Krebs buffer. Buffer was maintained at 37°C and continuously gassed with 95% O$_2$/5% CO$_2$. Rings were equilibrated for 30 minutes using a resting tension of 1g, during which the buffer was changed every 10 minutes. Dose response curves to AII were constructed by cumulative addition of small volumes of stock solution. Rings were maximally contracted with the thromboxane A2 (TxA2) analog U46619 (1µM).

**RNA isolation and cDNA synthesis**

Total RNA was isolated from frozen aortas using Tri-reagent (Molecular Research Center, Cincinnati, OH). Gel analysis confirmed RNA sample integrity by the presence of 18S and 28S ribosomal bands. First strand cDNA was synthesized from 1µg total RNA, using a RT-PCR CORE kit (Perkin Elmer, Norwalk, CT).

**Semi-quantitative PCR**

A semi-quantitative PCR analysis was performed to determine InsP$_3$R-1 and AT1R expression levels. During a semi quantitative polymerase chain reaction the mRNA of the gene of interest and the mRNA of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were coamplified in a single PCR reaction. A 50 µl PCR reaction mixture contained 0.5 units Taq polymerase, 5 µl of the supplied buffer, 17.5 nmol dNTP, 2 mM MgCl$_2$, 1 µl of
cDNA mixture and 40 pmol of each gene specific primers (Table 1) and 20 pmol of each GAPDH PCR primer (Table 1). Temperature cycling was performed in 0.5 ml thin-walled tubes in a thermal cycler (DNA thermal cycler, Perkin Elmer, Norwalk, CT) using a protocol of 30 cycles of 1 min. denaturation at 94 °C, 1 min. annealing at 56 °C and 1 min. extension at 72 °C. PCR products were separated on a 1.5% agarose gel by electrophoresis and stained with ethidium bromide. The gels were quantified by densitometry. The relative expression level of AT1R and InsP3R-1 mRNA was expressed as the ratio of InsP3R-1 or AT1R and GAPDH PCR products.

Table 1.
PCR primers

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<th>Primer Position:</th>
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<td>AT1-R</td>
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<td>Rat GAPDH</td>
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<td>620-645</td>
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Primers pairs used for the amplification of InsP3R-1, InsP3R-2, InsP3R-3, AT1R and GAPDH. The characters ‘B’ in primer Isotype-F and ‘S’ and ‘W’ in primer Isotype-R are IUPAC ambiguous codes (B= not A, S=C or G and W= A or T). Ambiguous nucleotides were used in order to obtain a complete match for each target template. The coordinates for localization refer to the sequences indicated by genbank accession numbers.

Ratio reverse transcriptase PCR
Ratio reverse transcriptase PCR (De Smedt et al., 1994) was used to determine the relative expression of InsP3R-1, InsP3R-2, InsP3R-3, AT1R and GAPDH. Using one primer set (Table 1), we were able to co-amplify all three InsP3R isoforms, taking advantage of the similarity of the primer-template sequences. A 50 µl PCR reaction mixture contained 0.5 units Taq polymerase, 5 µl of the supplied buffer, 17.5 nmol dNTP, 2 mM MgCl₂, 1 µl of cDNA mixture and 40 pmol of each primer. Temperature cycling was performed in 0.2 ml thin-walled tubes in a thermal cycler (DNA thermal cycler 9700, Perkin Elmer, Norwalk, CT) using
a protocol of 35 cycles of 30 sec. denaturation at 94 °C, 30 sec. annealing at 56 °C and 30 sec. extension at 72 °C. Radioactive labeling was done by diluting 20 µl of the reaction product in a new 50 µl PCR mixture containing 0.5 units Taq polymerase, 5 µl of the supplied buffer 2 mM MgCl₂ 40 pmol of each primer, supplemented with 10nCi/µl [α⁻³²P]dCTP. Incorporation of radiolabeled dCTP was achieved by performing 3 additional cycles. The amplified products were discriminated by digestion with restriction enzymes BclI and BglII. PCR products were cut overnight at 37°C and the fragments were separated on a 6% polyacrylamide gel and quantified by means of the PhosphorImager model 425 (Molecular Dynamics, CA).

Materials
Fresh solutions of AII were made daily from stock solutions. AII and U46619 were purchased from Calbiochem (San Diego, CA). STZ was obtained from Sigma (St. Louis, MO). The restriction enzymes BclI and BglII were purchased from Promega (Madison, WI). All PCR primers were synthesized by Eurogentec (Seraing, Belgium). The RT-PCR CORE kit and Taq DNA polymerase were purchased from Perkin Elmer (Norwalk, CT). [α⁻³²P]dCTP was obtained from Amersham (Arlington Heights, IL). Tri reagent was purchased from Molecular Research Center (Cincinnati, OH). All chemicals used were of analytical grade.

Statistics
Results are presented as mean ± s.e.mean unless indicated otherwise. Differences between concentration-response curves were analyzed using repetitive measurement ANOVA (SigmaStat 1.01, Jandel Scientific, Germany) and considered significant at p < 0.05. Differences in other variables were tested using Student t-test, or Chi-square test as indicated.

Results:
Body weight and blood glucose levels
Blood glucose levels (Table 2) were significantly higher (p<0.01) for both the diabetic rats and the anti-T treated diabetic rats. Anti-T treatment did not affect blood glucose levels of diabetic rats. Body weights at the time of experiments are shown in Table 2. Body weights of the diabetic rats were significantly lower than those of control animals (P<0.01). Body weights of anti-T treated diabetic rats were intermediate of those of diabetic and control animals, but did not differ significantly from either group.
Table 2
Summary of results.

<table>
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<th>Control (n=5)</th>
<th>Diabetic (n=6)</th>
<th>Diabetic Anti-T (n=5)</th>
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<tr>
<td>Body Weight (g)</td>
<td>268 ± 13</td>
<td>182 ± 13*</td>
<td>223 ± 19</td>
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<td>Blood sugar (mg/dl)</td>
<td>119 ± 12</td>
<td>470 ± 37*</td>
<td>486 ± 50*</td>
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<td>E\textsubscript{max} AII (% of U46619)</td>
<td>14.5 ± 2.1</td>
<td>7.4 ± 1.3*</td>
<td>12.6 ± 1.9</td>
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<td>EC50 AII (nM)</td>
<td>19.2 ± 2.9</td>
<td>24.8 ± 5.4</td>
<td>15.0 ± 4.1</td>
</tr>
<tr>
<td>E\textsubscript{max} U46619 (g)</td>
<td>1.2 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

AII E\textsubscript{max} values are expressed relative to the maximal contraction to U46619. *P < 0.05 vs. control, # P < 0.05 vs. diabetic.

Vasoconstrictive effect of AII on rat aortic rings
Cumulative dose-response curves to AII were constructed (Fig. 1) to investigate the effect of short-term diabetes on aortic ring responsiveness to AII. Responses are expressed relative to the maximal contraction to the thromboxane A2 analog, U46619. Maximal contraction to U46619 did not vary among groups (table 2). Dose-response curves to AII were significantly decreased in diabetic animals (Fig. 1). The EC50 values did not differ between groups (Table 2). Anti-T treatment of diabetic animals restored the AII induced aortic contractions to control levels.

FIG. 1 Dose-response curves of aortic rings of control animals (●), diabetic rats (■) and anti-T treated diabetic rats (▲) to angiotensin II. Relative maximal contractions for control animals, diabetic rats and anti-T treated diabetic rats were 14.5 ± 2.1%, 7.4 ± 1.3% and 12.6 ± 1.9%, respectively. Results are expressed as a percent of maximal contraction to u46619 and presented as mean ± s.e.mean. *P < 0.05 vs. control and anti-T treated diabetic rats.
InsP$_3$R isoform mRNA levels in rat aorta.

To investigate whether the impaired vascular contractility in diabetic animals is associated with changes in InsP$_3$R-1 expression, InsP$_3$R-1 mRNA levels were determined (Fig. 2A and Table 2). InsP$_3$R-1 mRNA levels were significantly decreased in diabetic animals compared to control animals. anti-T treatment restored InsP$_3$R-1 mRNA levels to those of control animals. GAPDH levels were identical for all groups.

FIG. 2 Bar graph showing InsP$_3$R-1 (A), InsP$_3$R-2 (B) and InsP$_3$R-3 (C) mRNA levels relative to GAPDH mRNA levels in rat aortic tissue. (A) InsP$_3$R-1 mRNA levels are significantly decreased in diabetic animals. anti-T treatment restored InsP$_3$R-1 mRNA levels to those of control animals. (B) InsP$_3$R-2 mRNA levels are not different between groups. (C) InsP$_3$R-3 mRNA levels are significantly decreased in diabetic animals when compared to control animals. InsP$_3$R-3 mRNA levels of the anti-T treated group are intermediate to those of control and diabetic animals, but did not differ significantly from either group. Results are presented as mean ± s.e.mean. *P < 0.05 vs. control.

To investigate whether the impaired vascular contractility in diabetic animals is associated with changes in the relative expression levels of InsP$_3$R isoforms, we performed ratio reverse transcriptase PCR (Fig. 3). InsP$_3$R-2 and InsP$_3$R-3 mRNA levels (Fig. 2B and 2C) were calculated by combining the data on the expression pattern of InsP$_3$R isoforms with the data on InsP$_3$R-1 mRNA levels. InsP$_3$R-2 mRNA levels did not differ between groups and was expression was much decreased in abundance in relation to InsP$_3$R-1 or InsP$_3$R-3. InsP$_3$R-3 mRNA levels were significantly decreased in diabetic animals when compared to control animals. InsP$_3$R-3 mRNA levels of the anti-T treated group were intermediate to those of control and diabetic animals, but did not differ significantly from either group.

AT1 receptor mRNA levels in rat aorta.

To investigate whether the impaired vascular contractility in diabetic animals is caused by downregulation of AT1 receptors, we determined AT1 receptor mRNA levels (Fig. 4). AT1R mRNA levels were significantly increased in
diabetic rats when compared to control animals. anti-T treatment resulted in a further increase of AT1R mRNA levels when compared to untreated diabetic rats (P<0.05).

**FIG. 3** Gel showing typical results for determination of the expression pattern of the three InsP3R isoforms in rat aorta tissue. Total RNA was reverse transcribed as described under “Methods”. Using a single primerset (Table 1), three cDNA targets with similar primer-template sequences were amplified. The amplified targets were discriminated by digestion with restriction enzymes with a cleavage site specific to InsP3R-1 and InsP3R-3. The intensities of the fragments were quantified by means of the PhosphorImager. Lane 1: undigested 295 bp InsP3R-1, InsP3R-2 and InsP3R-3 PCR product. Lane 2-4: double digestion with BclI and BglII. InsP3R-1 is cleaved into a 189 bp, 106 bp and a 6 bp fragment. InsP3R-2 is cleaved into a 289 bp and a 6 bp fragment. InsP3R-3 is cleaved into a 234 bp, a 55 bp and a 6 bp fragment.

**FIG. 4** Bar graph showing AT1R mRNA levels relative to GAPDH mRNA levels in rat aortic tissue. AT1R mRNA levels are significantly higher in diabetic rats when compared to control animals. anti-T treatment resulted in significantly higher AT1R mRNA levels when compared to untreated diabetic rats. *P < 0.05 vs. control. †P < 0.05 vs. diabetic rats.

**Discussion:**

In this study, we investigated whether TGF-β is involved in diabetes-induced changes in macrovascular tone. We found impaired aortic contractions to AII with no change in EC50 in diabetic animals. InsP3R-1 and InsP3R-3 mRNA levels were decreased whereas AT1R mRNA levels were increased in diabetic animals. Anti-T treatment restored AII mediated contractions and InsP3R-1 mRNA levels to control levels. anti-T treatment further increased AT1R mRNA levels. We suggest that hyperglycemia in diabetes attenuates the AII response through TGF-β mediated downregulation of InsP3R-1.

The impairment of aortic contractions to AII in diabetic animals is in good agreement with previously reported results of impaired AII contractions.
TGF-β antibody treatment...diabetic rats

(Beenen et al., 1996) and might be explained by an increase in basal NO production. Increased NO production has been reported in early diabetes (Bank and Aynedjian, 1993), although impaired basal NO production has also been found in later stages of streptozotocin induced diabetes in rats (Bolego et al., 1999). TGF-β has been found to inhibit synthesis and production of NO via the inducible form of nitric oxide synthase (Perrella et al., 1996b), however TGF-β may also enhance production of NO via the endothelial NOS (Ying and Sanders, 1998). If NO production was affected by anti-TGF-β antibodies it would be expected that an increase in basal NO production would also have affected U44619 mediated contractions, which was not the case in our study. The observation that U44619 mediated contraction remains intact despite reduced InsP3R levels suggests that this effect of U44619 is not solely dependent on InsP3-mediated calcium release. It has been previously reported that U44619 stimulates calcium influx independent of phospholipase C activation (Pulcinelli et al., 1998) and InsP3 production (Suganuma et al., 1992).

The restoration of impaired aortic contractions to AII in diabetic animals by anti-T treatment could be related to normalization of hyperglycemia or by restoring AT1 receptors. Although TGF-β may play a role in glucose uptake (Kitagawa et al., 1991) and insulin release (Sekine et al., 1994) anti-T treatment did not seem to affect the diabetic state per se, as blood D-glucose levels were identical for diabetic animals and antibody treated diabetic animals. Several studies have found altered expression of AII receptors in diabetes with inconclusive results (reviewed in (Brown and Sernia, 1994)). Our finding that the AT1a receptor mRNA is increased in the aorta of diabetic rats is consistent with the reported upregulation of AII receptors in cardiac, liver and adrenal gland tissue of STZ induced diabetic rats (Brown et al., 1997). The upregulation of AT1a receptor mRNA would suggest that down-regulation of AT1a receptor is not the cause of impaired AII vasoconstriction of aortic rings from diabetic rats. Whether the effect of anti-T to further increase AT1a receptor mRNA levels in the diabetic rats plays a role in restoring AII contraction remains possible.

Induction of diabetes resulted in a significant downregulation of InsP3R-1 and InsP3R-3 mRNA levels. The downregulation of InsP3R-1 is in good agreement with the downregulation of renal InsP3R-1 mRNA and protein in STZ induced diabetic rats and mice (Sharma et al., 1999). Although mRNA levels do not need to reflect protein expression, we previously demonstrated a good correlation between InsP3R-1 mRNA and InsP3R-1 protein levels (Sharma et
al., 1999) and between InsP$_3$R-1 promoter activity and InsP$_3$R-1 protein levels (Sipma et al., 1998). Therefore, it is likely that the downregulation of InsP$_3$R-1 mRNA levels reflects downregulation of InsP$_3$R-1 protein levels in aortic tissue of diabetic animals. The relation of InsP$_3$R levels and calcium release and contraction has been previously demonstrated. In cultured cells a clear relation exist between InsP$_3$R levels and Ca$^{2+}$ release (Sipma et al., 1998; Bokkala and Joseph, 1997). In cultured rat fetal aorta smooth muscle cells (A7r5 cells), a 40-50% reduction in InsP$_3$R-1 and InsP$_3$R-3 protein levels was associated with a 40-50% decrease in Ca$^{2+}$ release at 400 nM InsP$_3$ (Sipma et al., 1998). That aortic contraction is dependent on InsP$_3$R is supported by the observation that aortic contractions were impaired when InsP$_3$R’s were blocked by intracellular administration of the InsP$_3$R blocker heparin (Brailoiu et al., 1993). In our present study, downregulation of InsP$_3$R-1 and InsP$_3$R-3 mRNA levels (-25% and -38%, respectively) was associated with a 50% decrease in maximal contraction to AII. Therefore the magnitude of InsP$_3$R downregulation in diabetic animals is likely to have an marked effect on Ca$^{2+}$ homeostasis and contraction. Further, since InsP$_3$R mRNA levels were measured in aortic sections containing multiple cell types, the down-regulation of InsP$_3$R-1 and InsP$_3$R-3 mRNA levels in aortic sections may reflect an even larger down-regulation of InsP$_3$R mRNA levels in smooth muscle cells. From this, we suggest that the impaired aortic contraction to AII in diabetic rats is mediated, at least in part, by down-regulation of InsP$_3$R-1 and InsP$_3$R-3.

In our prior studies we had found that TGF-$\beta$ reduces InsP$_3$R-1 expression in mesangial cells (Sharma et al., 1997) and that suppression of InsP$_3$R-1 in the diabetic kidney was associated with up-regulation of TGF-$\beta$1 mRNA (Sharma et al., 1999). The results of the present study demonstrating that neutralization of TGF-b with antibodies restores the level of expression of InsP$_3$R-1 to control levels in diabetic rats is in good agreement with our previous investigations. The mechanisms underlying TGF-$\beta$ induced reduction of InsP$_3$R-1 is unclear but may be related to phosphorylation and proteasomal degradation at the protein level as well as transcriptional control at the level of the promoter. These studies are presently underway in our lab.

In summary, anti-T treatment to diabetic rats restored aortic responsiveness and InsP$_3$R-1 mRNA levels to nondiabetic control values. As the impaired contraction to AII is associated with downregulation of InsP$_3$R-1 expression and both contraction to AII and InsP$_3$R-1 expression is restored by anti-T, we conclude that TGF-$\beta$ mediated downregulation of InsP$_3$R-1 contributes to macrovascular dysfunction of diabetes.
References


TGF-β antibody treatment...diabetic rats


