Reactive oxygen species blockade and action of insulin on expression of angiotensinogen gene in proximal tubular cells

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Abstract

We reported previously that insulin inhibits the stimulatory effect of high glucose on the expression of angiotensinogen (ANG) gene in both rat immortalized renal proximal tubular cells (IRPTCs) and non-diabetic rat renal proximal tubular cells (RPTCs), but has no effect in diabetic rat RPTCs. In the present study we investigated whether hyperglycaemia-induced resistance to the insulin-induced inhibition of expression of the ANG gene is mediated via the generation of reactive oxygen species (ROS) in RPTCs. Rat IRPTCs were cultured for 2 weeks in high-glucose (25 mM) or normal-glucose (5 mM) medium plus angiotensin II (Ang II) with or without a superoxide scavenger (tiron), or inhibitors of: NADPH oxidase (diphenylene iodinium, DPI), Ang II type 1 and 2 receptors (losartan and PD123319), angiotensin-converting enzyme (perindopril), protein kinase C (GF 109203X), or glutamine:fructose-6-phosphate aminotransferase (azaserine). Cellular generation of ROS, and ANG and renin mRNA levels were assessed by lucigenin assay and specific reverse transcriptase-PCR respectively. Phosphorylation of p44/42 mitogen-activated protein kinase (p44/42 MAPK) was evaluated by western blotting. Prolonged exposure of IRPTCs to high concentrations of glucose or Ang II evoked generation of ROS and resistance to the insulin-induced inhibition of expression of the ANG gene and of p44/42 MAPK phosphorylation. Co-incubation of IRPTCs with tiron, DPI, losartan, PD123319, perindopril, GF 109203X or azaserine prevented ROS generation, restoring the inhibitory action of insulin on ANG gene expression and on p44/42 MAPK phosphorylation. In conclusion, our studies demonstrate that blockade of both ROS generation and activation of the intrarenal renin-angiotensin system improves the inhibitory action of insulin on ANG gene expression in IRPTCs in conditions of high glucose.


Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease (ESRD) in North America (Groggel 1996). Approximately 30–45% of all new cases of ESRD in Canada and the USA are attributed to diabetes (Berneger et al. 1994, United States Renal Data System 2003). The progression of diabetic nephropathy to ESRD is associated with kidney hypertrophy, proteinuria, tubulointerstitial fibrosis and cardiovascular diseases (Jensen et al. 1987, Parving et al. 1996). Studies over the past 10 years have demonstrated that intensive treatment with insulin and long-term treatment with renin–angiotensin system (RAS) blockers delay the progression of diabetic nephropathy, but do not cure it (Reichard et al. 1993, The Diabetes Control and Complications Trial Research Group 1993, 1995, 2000, Ravid et al. 1996, Andersen et al. 2000, Fliser & Haller 2001). Such results indicate, however, that hyperglycaemia and RAS activation are major determinants of diabetic nephropathy.

Angiotensinogen is the sole substrate of the RAS. It is now well accepted that there is a local intrarenal RAS (Dzau & Ingelfinger 1989, Johnston et al. 1993), and all components of the RAS are expressed in human, rat and murine renal proximal tubular cells (RPTCs) (Ingelfinger et al. 1990, Wolf & Neilson 1992, Anderson et al. 1993, Burns et al. 1993, Tang et al. 1995, Loghman-Adham et al. 1997, Lai et al. 1998, Wang et al. 1998, Zimpelmann et al. 2000).Angiotensin II (Ang II) concentrations and angiotensinogen and renin mRNA expression in the...
kidney are increased in early diabetes (Anderson et al. 1993, Lai et al. 1998, Zimpelmann et al. 2000), indicating that hyperglycaemia or augmented intrarenal Ang II, or both, may be directly or indirectly responsible for renal proximal tubular hypertrophy and tubulointerstitial fibrosis in diabetes. Indeed, in vitro studies have shown that the incubation of murine proximal tubular cells in high-glucose medium (25 mM) and high concentrations of Ang II (>10^{-8} M) induce cellular hypertrophy and extracellular matrix protein expression (Wolf & Neilson 1990, Ziyadeh et al. 1990, Wolf et al. 1991, 1993).

We have reported that high concentrations of glucose (25 mM) stimulate the expression of angiotensinogen protein and mRNA in rat immortalized RPTCs (Zhang et al. 1999b, 2000). RAS blockade and stable transfection of antisense rat angiotensinogen cDNA inhibited the effect of high glucose concentrations on the expression of angiotensinogen protein and mRNA, the expression of transforming growth factor-β1 mRNA and the induction of IRPTC hypertrophy (Zhang et al. 2001, 2002). More recently, we demonstrated that the stimulatory effect of high concentrations of glucose on the expression of ANG gene and IRPTC hypertrophy is mediated, at least in part, via generation of reactive oxygen species (ROS) and activation of both the hexosamine biosynthesis pathway (HBP) and protein kinase C (PKC) signalling (Hsieh et al. 2002, 2003). These investigations established that the intrarenal expression of ANG gene and subsequent RAS activation induced by high concentrations of glucose are essential for IRPTC hypertrophy.

We have also reported that insulin inhibits the stimulatory effect of high glucose concentrations on the expression of angiotensinogen protein and mRNA in both IRPTCs and non-diabetic rat RPTCs via the activation of p44/42 mitogen-activated protein kinase (p44/42 MAPK) (Zhang et al. 1999a, 2002a, b, Chen et al. 2001). In contrast, insulin did not suppress the expression of angiotensinogen protein and mRNA or activate p44/42 MAPK phosphorylation in diabetic rat RPTCs (Zhang et al. 2002a), suggesting that the effect of hyperglycaemia-induced insulin resistance on the inhibition of ANG gene expression is mediated, at least in part, via impairment of p44/42 MAPK activation in diabetic rat RPTCs. The underlying mechanism(s) of hyperglycaemia-induced insulin resistance, however, remain(s) undefined.

In the present study, we investigated the possible role(s) of ROS in mediating the role of high-glucose-induced insulin resistance in the inhibition of ANG gene expression in IRPTCs. Specifically, we aimed to determine whether prolonged exposure of IRPTCs to high concentrations of glucose for 2 weeks (that is, a situation that mimics early diabetes) could evoke insulin resistance in the inhibition of ANG gene expression and in activation of p44/42 MAPK in vitro. We also examined whether generation of ROS and subsequent activation of the RAS might mediate high-glucose induction of insulin resistance in the inhibition of ANG gene expression and the activation of p44/42 MAPK in IRPTCs. We found that prolonged exposure of IRPTCs to a high-glucose milieu or to Ang II in a normal-glucose milieu evoked generation of ROS and induced insulin resistance in the inhibition of ANG gene expression and in p44/42 MAPK activation. Co-incubation of IRPTCs with a superoxide scavenger, inhibitors of NADPH oxidase, RAS blockers, or inhibitors of PKC and HBP was effective in preventing the generation of ROS and, subsequently, in restoring the action of insulin in the inhibition of ANG gene expression and in p44/42 MAPK activation.

Materials and Methods

D(+)-Glucose, D-mannitol, L-glucose, glucosamine, human Ang II, phorbol 12-myristate 13-acetate (PMA) (a PKC activator), tiron (a superoxide scavenger), diphenyleneiodonium (DPI, an inhibitor of NADPH oxidase), PD123319 (an angiotensin II type 2 receptor (AT₂R) blocker), GF 109203X (a PKC inhibitor), azaserine [O-diazoacetyl-L-serine, an inhibitor of glutaminase: fructose-6-phosphate aminotransferase] and monoclonal antibodies against β-actin were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada). Losartan (a non-peptide angiotensin II type 1 receptor (AT₁R) blocker) and perindopril (an angiotensin-converted enzyme (ACE) inhibitor) were gifts from Dr Ronald D Smith (Dupont Merck, Wilmington, DE, USA) and Dr Serge Carrière (Servier Amérique, Laval, Quebec, Canada) respectively. Normal-glucose (1000 mg/l or 5 mM) Dulbecco’s Modified Eagle Medium (DMEM) (pH 7-45) (catalogue no. 12320-032), 100X penicillin/ streptomycin and fetal bovine serum (FBS) were obtained from Invitrogen, Inc. (Burlington, Ontario, Canada).

Oligonucleotides were synthesized by Invitrogen, Inc. Restriction and modifying enzymes were purchased from Invitrogen Inc., La Roche Biochemicals, Inc. (Laval, Quebec, Canada), or Amersham–Pharmacia Biotech. PhosphoPlus p44/42 MAPK antibody kits from New England Biolabs, Inc. (Mississauga, Ontario, Canada) were used for the rapid analysis of p42/44 MAPK (Thr^{202}/ Tyr^{204}) phosphorylation status in the MAPK cascade.

Cell culture

IRPTCs (cell line no. 93-p-2-1) at passages 18 to 25 were used in the present study. The characteristics of this cell line have been described previously (Ingelfinger et al. 1999). These cells express the mRNA and protein of angiotensinogen, renin, ACE and Ang II receptors (both AT₁R and AT₂R) (Ingelfinger et al. 1999).

IRPTCs were grown in 100 × 20 mm plastic Petri dishes (Invitrogen, Inc.) in normal-glucose (5 mM) DMEM (pH 7-45), supplemented with 5% FBS, 100 U/ml...
penicillin, and 100 µg/ml streptomycin, in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. For subculturing, cells were trypsinized (0·05% trypsin and EDTA) and plated at 2·5 × 10⁴ cells/cm² in 100 × 20 mm Petri dishes.

**Effect of high glucose and Ang II on ROS generation in the absence or presence of superoxide scavenger, RAS blockers, or inhibitors of NADPH oxidase, PKC and HBP in IRPTCs**

ROS production was measured by the lucigenin method (Grindling et al. 1994) with minor modifications, as described previously (Hsieh et al. 2002). Briefly, the cells were incubated in high-glucose (25 mM) DMEM and 5% FBS or in normal-glucose (5 mM) DMEM containing Ang II (10⁻⁷ M) and 5% FBS for 48 h or 2 weeks in the absence or presence of DPI (1 × 10⁻⁶ M), tiron (1 × 10⁻⁴ M), losartan (1 × 10⁻⁶ M), PD123319 (1 × 10⁻⁶ M), perindopril (1 × 10⁻⁴ M), GF 109203X (1 × 10⁻⁷ M) or azaserine (1 × 10⁻⁷ M). The cells were then trypsinized, collected by centrifugation, and the pellet washed in modified Krebs buffer containing NaCl (130 mM), KCl (5 mM), MgCl₂ (1 mM), CaCl₂ (1·5 mM), K₂HPO₄ (1 mM) and Hepes (20 mM), pH 7·4. After washing, the cells were resuspended in Krebs buffer with 1 mg/ml BSA, and cell concentration was adjusted to 1 × 10⁶ in 900 µl buffer. To measure ROS production, the cell suspension was transferred into plastic tubes and assessed in a luminometer (LB 9507; Berthold, Wildbad, Germany). Measurement was started by an injection of 100 µl lucigenin (final concentration 5 × 10⁻⁴ M). Photon emission was counted every 1 min for up to 20 min. Modified Krebs buffer was used as control (blank).

**Effect of insulin on expression of angiotensinogen mRNA in IRPTCs in the absence or presence of superoxide scavenger, RAS blockers, or inhibitors of NADPH oxidase, PKC and HBP**

IRPTCs were cultured for 2 weeks in high-glucose DMEM containing 5% FBS in the absence or presence of DPI (1 × 10⁻⁶ M), tiron (1 × 10⁻⁴ M), losartan (1 × 10⁻⁶ M), PD123319 (1 × 10⁻⁶ M), perindopril (1 × 10⁻⁴ M), GF 109203X (1 × 10⁻⁷ M) or azaserine (1 × 10⁻⁷ M). Alternatively, IRPTCs were cultured for 2 weeks in normal-glucose DMEM containing 5% FBS in the absence or presence of Ang II (1 × 10⁻⁷ M), PMA (1 × 10⁻⁷ M) or glucosamine (1 × 10⁻⁶ M). The media were changed every 24 h. After a 2-week incubation period, cell growth was arrested (that is, cells were synchronized in the same phase of the cell cycle) by

**Figure 1** Effect of Ang II on generation of ROS in rat IRPTCs in the absence or presence of inhibitors. (A) Cells incubated in 5 mM D-glucose medium for 48 h in the absence or presence of various concentrations of Ang II. (B) Cells preincubated in 5 mM D-glucose DMEM containing Ang II (1 × 10⁻⁷ M) and 5% FBS for 48 h in the absence or presence of tiron (1 × 10⁻⁴ M), DPI (1 × 10⁻⁶ M), losartan (1 × 10⁻⁶ M), PD123319 (1 × 10⁻⁶ M) or perindopril (1 × 10⁻⁴ M). (C) Cells preincubated in 25 mM D-glucose DMEM containing 5% FBS for 48 h in the absence or presence of tiron (1 × 10⁻⁴ M), DPI (1 × 10⁻⁶ M), losartan (1 × 10⁻⁶ M), PD123319 (1 × 10⁻⁶ M) or perindopril (1 × 10⁻⁴ M). The cells were then trypsinized for the assessment of ROS generation. Measurements of emission of relative light units (RLU), corrected for non-specific luminescence in the absence of cells, were compared after 10 min of incubation. Cells incubated in 5 mM D-glucose medium were considered as controls (100%). Each point represents the mean ± s.d. of four independent experiments. *P<0.05, **P<0.01, N.S., not significant.
incubation in serum-free medium with normal-glucose DMEM for 24 h. The cells were then incubated in normal- or high-glucose culture medium containing 1% depleted FBS (dFBS) in the absence or presence of insulin (1 × 10^{-7} M) for an additional 24 h. At the end of the incubation period, the cells were harvested and total RNA was extracted and assayed for angiotensinogen, renin and β-actin mRNA levels, as described previously (Hsieh et al. 2002, 2003).

Total RNA (2 µg) was used to synthesize first-strand cDNAs in the Super-Script pre-amplification system (Invitrogen, Inc.). The first-strand cDNAs were then diluted with water to a ratio of 1:4 and aliquots processed to amplify rat angiotensinogen, renin and β-actin cDNA fragments with the polymerase chain reaction (PCR) core kit (Invitrogen, Inc.). First-strand cDNAs (5 µl) and primers of rat angiotensinogen, renin (400 nM) or rat β-actin (100 nM) were added in a final volume of 50 µl PCR mixture (final concentration: 1 × PCR buffer, 0·2 mM dNTP, 1·5 mM MgCl₂, and 2·0 units Taq DNA polymerase) (Invitrogen, Inc.). The PCR mixture was amplified in a RapidCycler (Idaho Technology Inc., Salt Lake City, Utah, USA). After denaturation at 94 °C for 3 min, angiotensinogen, renin and β-actin cDNAs were co-amplified in the same tube under the following conditions: 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 40 s. After 30 cycles, PCRs were further extended at 72 °C for 5 min.

The sense and antisense rat angiotensinogen primers used were 5'-CCT CGC TCT CTG GAC TTA TC-3' and 5'-CAG ACA CTG AGG TGC TGT TG-3', corresponding to nucleotide sequences N+676 to N+695 and N+882 to N+901 of rat angiotensinogen cDNA (Ohkubo et al. 1983) respectively. The sense and antisense rat renin primers were 5'-CTG CCA CCT TGT TGT GTG AG-3' and 5'-CCA GTA TGC ACA GGT CAT CG-3', corresponding to the nucleotide sequences N+1033 to N+1052 and N+1277 to N+1296 of rat renin cDNA (Burnham et al. 1997) respectively. The sense and antisense rat β-actin primers were 5'-ATG CCA TCC TGC GTC TGG ACC TGG C-3' and 5'-AGC ATT TGC GGT GCA CGA TGG AGG G-3', corresponding to nucleotide sequences N+155 to N+179 of exon 3, and nucleotide sequences N+115 to N+139 of exon 5 of the rat β-actin gene (Nudel et al. 1983) respectively.

To identify rat angiotensinogen, renin and β-actin cDNA fragments, 10 µl PCR products were electrophoresed on 1·2% agarose gel and transferred onto a Hybond-N+ nylon membrane (Amersham-Pharmacia Biotech). Digoxigenin (DIG)-labelled oligonucleotides 5'-GAG GGG GTC AGC ACG GAC AGC ACC-3' and 5'-TCC TGC GTG ACC TGG C-3' and 5'-AGC ATT TGC GGT GCA CGA TGG AGG G-3', corresponding to nucleotide sequences N+775 to N+798 of rat angiotensinogen cDNA (Ohkubo et al. 1983) and nucleotides N+1119 of rat renin cDNA (Burnham et al. 1997) respectively.

Figure 2 Effect of prolonged exposure of IRPTCs to Ang II, PMA or high glucose concentrations on production of ROS in the absence or presence of a superoxide scavenger, inhibitors of NAPDH oxidase, PKC and HBP or RAS blockers. (A) Cells preincubated in 5 mM d-glucose DMEM containing Ang II (1 × 10^{-7} M) or PMA (1 × 10^{-7} M) and 5% FBS, or in high-glucose medium and 5% FBS for 2 weeks. (B) Cells preincubated in 25 mM d-glucose DMEM in the absence or presence of tiron (1 × 10^{-6} M), DPI (1 × 10^{-6} M), losartan (1 × 10^{-6} M), PD123319 (1 × 10^{-6} M), perindopril (1 × 10^{-6} M), GF 109203X (1 × 10^{-6} M) or azaserine (AZA; 1 × 10^{-7} M). The cells were then trypsinized for the assessment of ROS generation. Relative light unit (RLU) measurements were compared after 10 min of incubation. Cells incubated in 5 mM d-glucose medium were considered as controls (100%). Each point represents the mean ± s.d. of four independent experiments. *P<0·05, **P<0·01.
to N+1139 of rat renin cDNA (Burnham et al. 1997) respectively, prepared with a DIG oligonucleotide 3'-end labelling kit (La Roche Biochemicals, Inc.), were used to hybridize PCR products on the membrane. After stringent washing, the membrane was processed with a DIG luminescent detection kit (La Roche Biochemicals, Inc.) and exposed to Hyperfilm MP (Amersham-Pharmacia Biotech). After angiotensinogen and renin mRNA analysis, the same membrane was stripped and rehybridized with a β-actin oligonucleotide probe (sequence: 5'TCC TGT GGC ATC CAT GAA ACT ACA TTC-3', corresponding to nucleotides N+9 to N+35 of exon 4 of the rat β-actin gene (Nudel et al. 1983)). Angiotensinogen and renin mRNAs were standardized against corresponding β-actin mRNA levels.

To maintain constant isotonicity or osmolality, the 5 mM glucose media were supplemented with D-mannitol (final concentration 20 mM).

dFBS was prepared by incubation with 1% activated charcoal and 1% AG 1X 8 ion-exchange resin (Bio-Rad Laboratories, Richmond, CA, USA) for 16–24 h at room temperature as described by Samuels et al. (1979), a procedure that removes endogenous steroid and thyroid hormones from FBS.

**Effect of insulin on p44/42 MAPK activation in IRPTCs in the absence or presence of superoxide scavenger, RAS blockers, or inhibitors of NADPH oxidase, PKC and HBP**

The effect of insulin on p44/42 MAPK activation in IRPTCs was assessed by western blotting with the PhosphoPlus p44/42 MAPK antibody kit. Briefly, IRPTCs were cultured for 2 weeks in high-glucose medium and 5% FBS in the presence or absence of DPI (1×10⁻⁶ M), tiron (1×10⁻⁴ M), losartan (1×10⁻⁶ M), PD123319 (1×10⁻⁶ M), perindopril (1×10⁻⁴ M), GF 109203X (1×10⁻⁷ M) or azaserine (1×10⁻⁷ M). Alternatively, IRPTCs were cultured for 2 weeks in normal-glucose medium and 5% FBS in the absence or presence of Ang II (1×10⁻⁷ M), PMA (1×10⁻⁷ M) or glucosamine (1×10⁻⁶ M). Cell growth was arrested by incubating the cells in serum-free medium with normal-glucose DMEM for 24 h. The cells were then incubated in normal glucose or high glucose DMEM containing 1% dFBS in the absence or presence of insulin (1×10⁻⁷ M) for 10 min, followed by harvesting and assay for p44/42 MAPK phosphorylation, as described previously (Zhang et al. 2002a, b).

Briefly, the cells were lysed in 700 µl lysis buffer (62.5 mM Tris–HCl, pH 6.8, containing 2% (wt/vol) SDS, 10% glycerol, 50 mM dithiothreitol, 1 mM phenyl methylsulphonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 0.1% (wt/vol) bromphenol blue), and transferred into Eppendorf tubes. The cell lysates were sonicated for 15 s, heated at 95 °C for 5 min and centrifuged at 12 000 g for 5 min. Thirty-five-microtitre aliquots

Figure 3 Effect of Ang II, PMA, glucosamine and RAS blockers on basal expression of angiotensinogen mRNA in IRPTCs. (A) Cells cultured for 2 weeks in 5 mM glucose medium and 5% FBS in the presence of Ang II (1×10⁻² M), PMA (1×10⁻⁷ M) or glucosamine (GlcN; 1×10⁻⁶ M). (B) Cells cultured in 25 mM glucose medium and 5% FBS in the presence or absence of losartan, PD123319 or perindopril. Cells were collected and assayed for rat angiotensinogen (rANG) and β-actin mRNA by RT-PCR assays, as described in Materials and Methods. The relative densities of angiotensinogen were standardized against the β-actin control. Angiotensinogen mRNA levels in IRPTCs incubated in 5 mM D-glucose DMEM were considered as controls (100%). Each point represents the mean ± s.d. of four independent experiments. **P<0.01, ***P<0.005.
of the supernatants were subjected to SDS–10% PAGE, then transferred onto a polyvinylidene difluoride membrane (Hybond-P, Amersham–Pharmacia Biotech). The membrane was initially blotted for phosphorylated p44/42 MAPK and then re-blotted for total p44/42 MAPK, according to the supplier’s instructions (New England Biolabs, Inc.).

Statistical analysis
Four or five separate independent experiments were performed per procedure, and each treatment group was run in duplicate. The data were analysed by one-way analysis of variance and the Bonferroni test. A probability level of \( P<0.05 \) was regarded as statistically significant.

Results

Effect of high glucose and Ang II on ROS generation in IRPTCs in the absence or presence of superoxide scavenger, RAS blockers, or inhibitors of NADPH oxidase, PKC and HBP
Lucigenin assay revealed that Ang II evoked the generation of ROS in IRPTCs in a dose-dependent manner, with a maximal effect at \( 1 \times 10^{-7} \) M (a more than two-fold increase) after 48 h incubation, compared with cells in normal-glucose medium without Ang II (Fig. 1A). The addition of tiron \((1 \times 10^{-6} \text{ M})\), DPI \((1 \times 10^{-6} \text{ M})\), losartan \((1 \times 10^{-6} \text{ M})\), or PD123319 \((1 \times 10^{-6} \text{ M})\) to the culture medium abolished the Ang II-stimulated generation of ROS, but perindopril had no significant effect (Fig. 1B). Similarly, the addition of tiron \((1 \times 10^{-6} \text{ M})\), DPI \((1 \times 10^{-6} \text{ M})\), losartan \((1 \times 10^{-6} \text{ M})\), PD123319 \((1 \times 10^{-6} \text{ M})\) or perindopril \((1 \times 10^{-6} \text{ M})\) abolished the stimulatory effect of high glucose concentrations on generation of ROS (Fig. 1C). Generation of ROS was also significantly greater in IRPTCs incubated for 2 weeks in normal-glucose medium in the presence of Ang II \((1 \times 10^{-7} \text{ M})\) or PMA \((1 \times 10^{-7} \text{ M})\) and in cells incubated in high-glucose medium for 2 weeks, compared with cells cultured in normal-glucose medium (Fig. 2A). The addition of DPI \((1 \times 10^{-6} \text{ M})\), tiron \((1 \times 10^{-6} \text{ M})\), losartan \((1 \times 10^{-6} \text{ M})\), PD123319 \((1 \times 10^{-6} \text{ M})\) or perindopril \((1 \times 10^{-6} \text{ M})\) abolished the stimulatory effect of high glucose on ROS generation (Fig. 2B). These results indicate that Ang II could evoke the generation of ROS in IRPTCs via both AT1R and AT2R and subsequent activation of NADPH oxidase activity. Thus high-glucose induction of the generation of ROS appeared to be mediated, at least in part, via activation of the intrarenal RAS.

Figure 4 Effect of prolonged exposure to high concentrations of glucose on basal expression of angiotensinogen and renin mRNA in IRPTCs in the absence or presence of a superoxide scavenger or inhibitors of NADPH oxidase, PKC and HBP. Cells were cultured for 2 weeks in 25 mM glucose medium and 5% FBS in the presence of tiron \((1 \times 10^{-6} \text{ M})\), DPI \((1 \times 10^{-6} \text{ M})\), losartan \((1 \times 10^{-6} \text{ M})\), PD123319 \((1 \times 10^{-6} \text{ M})\) or azaserine \((1 \times 10^{-6} \text{ M})\) or azaserine \((1 \times 10^{-7} \text{ M})\) or perindopril \((1 \times 10^{-6} \text{ M})\) abolished the stimulatory effect of high glucose on ROS generation. The relative densities of angiotensinogen mRNA (A) or renin mRNA (B) and \( \beta \)-actin mRNA by RT-PCR assays, as described in Materials and Methods. Angiotensinogen mRNA or renin mRNA levels in IRPTCs incubated in 5 mM D-glucose DMEM plus 20 mM D-mannitol were considered as controls (100%). Each point represents the mean ± S.D. of four independent experiments. **\( P<0.01 \), ***\( P<0.005 \).
Effect of high glucose and Ang II on angiotensinogen and renin mRNA expression in IRPTCs in the absence or presence of superoxide scavenger, RAS blockers, or inhibitors of NADPH oxidase, PKC and HBP

Figure 3A shows that prolonged incubation (2 weeks) of IRPTCs in normal-glucose medium in the presence of AngII (1 × 10^{-7} M), PMA (1 × 10^{-7} M) or glucosamine (1 × 10^{-6} M) or in high-glucose medium enhanced by two- to threefold the increase in expression of angiotensinogen mRNA compared with that in controls (cells incubated in 5 mM glucose medium). In contrast, incubation of IRPTCs in high-glucose medium for 2 weeks in the presence of losartan (1 × 10^{-6} M), PD123319 (1 × 10^{-6} M) or perindopril (1 × 10^{-4} M) prevented the stimulatory effect of high glucose on the expression of angiotensinogen mRNA in IRPTCs (Fig. 3B). Similarly, incubation of IRPTCs in high-glucose medium for 2 weeks in the presence of DPI (1 × 10^{-6} M), tiron (1 × 10^{-4} M), GF 109203X (1 × 10^{-6} M) or azaserine (1 × 10^{-7} M) prevented the stimulatory effect of high glucose on the expression of renin mRNA (Fig. 4A). Interestingly, incubation of IRPTCs in high-glucose medium enhanced basal expression of renin mRNA compared with the controls (normal-glucose medium) (Fig. 4B). The stimulatory effect of high glucose concentrations on expression of renin mRNA was inhibited in the presence of DPI (1 × 10^{-6} M), tiron (1 × 10^{-4} M), perindopril (1 × 10^{-6} M) or losartan (1 × 10^{-6} M).
These data suggest that the stimulatory action of high concentrations of glucose on the expression of angiotensinogen and renin mRNA in IRPTCs is mediated, at least in part, via generation of ROS, PKC and HBP signalling and, subsequently, RAS activation.

Effect of Ang II-, PMA- and glucosamine-induced insulin resistance on the inhibition of angiotensinogen mRNA expression in IRPTCs

Figure 5A reveals that high concentrations of glucose stimulated, whereas insulin inhibited, the expression of angiotensinogen mRNA in IRPTCs that had been cultured for 2 weeks in normal-glucose medium. Neither high glucose nor insulin, however, had any effect on the expression of angiotensinogen mRNA in IRPTCs that had been cultured for 2 weeks with Ang II (1 × 10^{-7} M) (Fig. 5B), PMA (1 × 10^{-7} M) (Fig. 5C) or glucosamine (1 × 10^{-6} M) (Fig. 5D) in normal-glucose medium. These data indicate that prolonged exposure to Ang II, PKC or HBP activator induced insulin resistance in the inhibition of expression of angiotensinogen mRNA in IRPTCs.

Effect of ROS and RAS blockade on the action of insulin in inhibition of angiotensinogen mRNA expression in IRPTCs in high-glucose medium

Figure 6A shows that neither high glucose concentrations nor insulin had any effect on the expression of angiotensinogen mRNA in IRPTCs in high-glucose medium. After incubation for 2 weeks in 25 mM glucose medium alone (A), or in the presence of (B) losartan (1 × 10^{-6} M), (C) PD123319 (1 × 10^{-6} M) or (D) perindopril (1 × 10^{-4} M), the cells were synchronized for 24 h in serum-free medium, then incubated for an additional 24 h in 5 mM or 25 mM glucose medium in the absence or presence of insulin (1 × 10^{-7} M), harvested and assayed for levels of angiotensinogen (rANG) and β-actin mRNA by RT-PCR, as described in Materials and Methods. The relative densities of angiotensinogen mRNA were standardized against the β-actin mRNA control. Angiotensinogen mRNA levels in IRPTCs incubated in 5 mM d-glucose DMEM plus 20 mM d-mannitol were considered as controls (100%). Each point represents the mean ± S.D. of four independent experiments in duplicate. *P<0.05, **P<0.01, ***P<0.005, N.S., not significant.
angiotensinogen mRNA in IRPTCs that had been incubated for 2 weeks in high-glucose medium. Co-incubation with losartan (1 × 10⁻⁶ M), PD123319 (1 × 10⁻⁶ M), or perindopril (1 × 10⁻⁵ M) restored the stimulatory and inhibitory action of high glucose concentrations and insulin on the expression of angiotensinogen mRNA in IRPTCs (Fig. 6B, C and D respectively). Similarly, co-incubation with tiron (1 × 10⁻⁴ M), DPI (1 × 10⁻⁶ M), GF 109203X (1 × 10⁻⁷ M), or azaserine (1 × 10⁻⁷ M) also restored the stimulatory and inhibitory action of high glucose concentrations and insulin on the expression of angiotensinogen mRNA in IRPTCs (Fig. 7A–D respectively). These data affirm that high-glucose induction of insulin resistance in the inhibition of expression of angiotensinogen mRNA is probably mediated via ROS generation, PKC and HBP activation, and subsequent RAS activation in IRPTCs.

Effect of Ang II, PMA and glucosamine-induced insulin resistance on phosphorylation of p44/42 MAPK in IRPTCs

Figure 8A illustrates that insulin stimulated the phosphorylation of p44/42 MAPK in IRPTCs that were cultured for 2 weeks in normal-glucose medium, whereas high glucose concentrations had no significant stimulatory effect. In contrast, neither high glucose nor insulin had any influence on p44/42 MAPK phosphorylation in IRPTCs that were cultured for 2 weeks in normal-glucose medium.
in the presence of Ang II (1 × 10⁻⁷ M) (Fig. 8B), PMA (1 × 10⁻⁷ M) (Fig. 8C) or glucosamine (1 × 10⁻⁶ M) (Fig. 8D). These data demonstrate that prolonged incubation with Ang II, PKC or HBP activator impairs the effect of insulin signalling on p44/42 MAPK activation in IRPTCs.

**Effect of ROS and RAS blockade on insulin signalling in the phosphorylation of p44/42 MAPK in IRPTCs in high-glucose medium**

Figure 9A shows that neither high glucose concentrations nor insulin exerted any effect on p44/42 MAPK phosphorylation in IRPTCs that had been incubated for 2 weeks in high-glucose medium. Co-incubation with losartan (1 × 10⁻⁶ M), PD123319 (1 × 10⁻⁶ M), or perindopril (1 × 10⁻⁴ M) restored the insulin signalling in the phosphorylation of p44/42 MAPK in IRPTCs in high-glucose medium (Fig. 9B–D respectively). Similarly, co-incubation with tiron (1 × 10⁻⁴ M), DPI (1 × 10⁻⁷ M), GF 109203X (1 × 10⁻⁷ M) or azaserine (1 × 10⁻⁷ M) also restored the action of insulin on p44/42 MAPK phosphorylation in IRPTCs (Fig. 10A–D respectively). These data demonstrate that ROS and RAS blockade and inhibition of PKC and HBP signalling are effective in restoring insulin signalling in the phosphorylation of p44/42 MAPK in IRPTCs in high-glucose medium.

**Discussion**

The findings of the present study demonstrate that ROS blockade and inhibition of intrarenal RAS activation restore the action of insulin in the inhibition of expression of the ANG gene and in the activation of p44/42 MAPK in IRPTCs in high-glucose medium. Our data suggest...
that ROS and intrarenal RAS activation may mediate the induction of insulin resistance by high glucose in the expression of the ANG gene in diabetic rat RPTCs.

The beneficial effects of inhibition of the RAS in the progression of diabetic nephropathy, hypertension and cardiovascular diseases have been reported in many clinical trials (Shieh et al. 1992, Torlone et al. 1993, Raccah et al. 1994, Mohanran & Toto 2003). Ang II has been found to attenuate or inhibit insulin signalling in vascular smooth muscle cells (Vellosa et al. 1996, Folli et al. 1997, Fukuda et al. 2001, Motley et al. 2003), implicating a role for the RAS in regulating the actions of insulin. Recent experiments have shown that Ang II is a potent inducer of renal oxidative stress, both in vivo and in vitro (Hannken et al. 1998, James et al. 1998, Haugen et al. 2000). Furthermore, hydrogen peroxide attenuates insulin signalling in cultured vascular cells and adipocytes (Rudich et al. 1997, Gardner et al. 2003), whereas lipoic acid protects against oxidative-stress-induced impairment of insulin signalling (Rudich et al. 1999, Maddux et al. 2001). Taken together, such findings suggest that there might be a link between Ang II, oxidative stress and insulin resistance.

The present study showed that Ang II, PMA and high glucose concentrations evoke generation of ROS and enhance the expression of the ANG mRNA gene in IRPTCs (Hsieh et al. 2002, 2003). These data also support the notion that the stimulatory effect of high glucose concentrations on the expression of ANG mRNA gene in IRPTCs (Hsieh et al. 2002, 2003).
angiotensinogen mRNA is probably mediated via local Ang II in IRPTCs, probably involves both AT1R and AT2R, enhances the generation of ROS, and subsequently activates both PKC and the HBP. Furthermore, our present findings confirm those of previous studies (Ingelfinger et al. 1999, Zhang et al. 2001) showing that Ang II exerts ‘positive’ feedback on the expression of angiotensinogen mRNA in RPTCs, both in vitro and in vivo.

Finally, it is noteworthy that prolonged exposure of IRPTCs to high concentrations of glucose for 2 weeks induced cellular hypertrophy, and RAS blockers reversed this effect of high glucose (unpublished results), consistent with high-glucose-induced IRPTC hypertrophy after 48 h of incubation (Zhang et al. 2001).

Surprisingly, PD123319 also inhibited the stimulatory effect of high concentrations of glucose on the expression of angiotensinogen mRNA in IRPTCs, suggesting that the stimulatory action of Ang II on the expression of angiotensinogen mRNA could be mediated, at least in part, via both AT1R and AT2R in RPTCs. To the best of our knowledge, this is the first report of AT2R blockade attenuating the expression of ANG gene in RPTCs in high-glucose media; the importance of this finding is unclear. Only a very low level of expression of AT2R is detected in the adult kidney (Shanmugam et al. 1995, Ozone et al. 1997). AT2R is re-expressed, however, in proximal tubules after ischaemia (Kontogiannis & Burns 1998) and in human diabetic nephropathy (Mezzano et al. 2003). We have previously reported the expression of AT2R in IRPTCs (Tang et al. 1995). Our recent studies (unpublished results) also showed that expression of AT2R is augmented in spontaneously diabetic BioBreeding rat
renal proximal tubules. These results suggest that AT$_2$R, like AT$_1$R, might exert autocrine-positive feedback on the expression of angiotensinogen mRNA and activation of the RAS, and subsequently modulate IRPTC function.

High-glucose-stimulated expression of renin mRNA and its effect were inhibited by tiron, DPI, GF 109203X and azaserine. These findings support the observations of Kelly et al. (1998) and Zimpelmann et al. (2000) that renin activity and renin mRNA are augmented in renal proximal tubules in early diabetes. Superoxide scavengers and inhibitors of NAPDH oxidase, PKC or HBP prevent the stimulatory effect of high glucose concentrations on the expression of renin mRNA – a novel finding. Thus our data suggest that high concentrations of glucose could directly activate the intrarenal RAS to yield Ang II and subsequently affect the generation of ROS in IRPTCs.

Insulin suppressed the acute stimulatory effect of high glucose on angiotensinogen mRNA in IRPTCs that had been cultured for 2 weeks in normal-glucose medium. Prolonged exposure of IRPTCs to Ang II, PMA, glucosamine or high concentrations of glucose induced resistance to the inhibitory effect of insulin on the expression of angiotensinogen mRNA in IRPTCs, whereas incubation of IRPTCs in high-glucose medium with antioxidants and inhibitors of RAS and HBP restored the acute stimulatory effect of high glucose concentrations and the inhibitory action of insulin on angiotensinogen mRNA in IRPTCs. These data imply that intrarenal generation of ROS and activation of the RAS are key players in the induction of resistance to the inhibitory action of insulin on ANG gene expression.

High concentrations of glucose failed to stimulate the expression of angiotensinogen mRNA in cells that had been cultured for 2 weeks with Ang II or PMA. The reasons for this are unclear; one possibility is that prolonged exposure to Ang II or PMA downregulated PKC activity and levels of expression of protein (Hug & Sarre 1993). Likewise, the mechanism(s) underlying the lack of influence of insulin on expression of the ANG gene in cells that had been cultured with Ang II or PMA is unclear. It is possible that activated PKC isoform(s) impairs insulin signalling by increasing the phosphorylation of insulin receptor substrates (IRS)-1/2 at serine/threonine residues. Indeed, PKC isoform(s) attenuates insulin signalling by IRS-1 phosphorylation at serine/threonine residues (Chin et al. 1994, Bossenmaier et al. 1997, De Fea & Roth 1997, Strack et al. 2000). Therefore, activation of PKC isoform(s) might be an important negative regulator of insulin receptor function. Most importantly, AT$_2$R activation may have a pivotal role in this cascade by mobilizing PKC signalling.

It is interesting that incubation of IRPTCs with glucosamine induced insulin resistance in the inhibition of expression of angiotensinogen mRNA, and incubation of IRPTCs in high-glucose medium with azaserine restored the action of insulin in inhibiting the expression of angiotensinogen mRNA. These data suggest that activation of HBP could elicit an insulin resistance in the inhibition of expression of angiotensinogen mRNA in IRPTCs similar to that observed in skeletal muscle and adipose tissue (Cooksey & McClain 2002, Chen et al. 2003, Spampinato et al. 2003). However, the molecular mechanism(s) by which HBP induces insulin resistance in the inhibition of expression of angiotensinogen mRNA remains to be defined.

Confirming the findings of previous studies (Zhang et al. 1999a, 2002b, Chen et al. 2001), we also found that insulin stimulated the phosphorylation of p44/42 MAPK in IRPTCs that had been incubated in normal-glucose medium. In contrast, prolonged exposure to Ang II, PMA, glucosamine or high concentrations of glucose blocked the action of insulin on the phosphorylation of p44/42 MAPK in IRPTCs. Co-incubation with losartan, PD123319, perindopril, tiron, DPI, GF 109293X or azaserine restored the effect of insulin on p44/42 MAPK phosphorylation in high-glucose medium. Thus these data further support our hypothesis that high-glucose-induced resistance to the inhibitory effect of insulin on the expression of the ANG gene is mediated, at least in part, via the generation of ROS, PKC and HBP signalling and subsequent activation of the RAS, eventually impairing the insulin signalling pathway in p44/42 MAPK phosphorylation.

In summary, the present study has shown that prolonged exposure of IRPTCs to high concentrations of glucose or to Ang II in normal-glucose medium induces resistance to the inhibitory effect of insulin on expression of angiotensinogen mRNA. The effect of high glucose concentrations was reversed by ROS and RAS blockade. These results suggest that intrarenal generation of ROS and activation of the RAS may contribute to the development of resistance to insulin inhibition of expression of the ANG gene in early diabetic IRPTCs. Our findings raise the possibility that antioxidants might be useful in improving the insulin sensitivity of suppression of local renal RAS activation and subsequently ameliorate renal injury in diabetes. Such an approach needs to be explored as a novel addition to other renoprotective therapies.

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