Oxidative stress impairs insulin internalization in endothelial cells in vitro

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Abstract

Aims/hypothesis. Because oxidative stress has been suggested to be a significant contributing factor in the development of endothelial dysfunction and insulin resistance, we investigated whether reactive oxygen species contribute to insulin resistance by impairing insulin uptake through an effect on endothelial insulin receptor function.

Methods. Following a 2-h pro-oxidant challenge with xanthine oxidase, we examined the temporal pattern of insulin processing in the human umbilical endothelial cell line Ea.Hy926 and bovine aortic endothelial cells equilibrated with [125 I]-insulin. Insulin receptor mRNA concentrations were analysed by RT-PCR and insulin receptor tyrosine phosphorylation and protein concentrations were estimated by western blotting.

Results. Xanthine oxidase exposure resulted in a major reduction in total insulin receptor-mediated [125 I]-insulin internalization over a 1-h period in both Ea.Hy926 and bovine aortic endothelial cells. After 15 min, untreated bovine aortic endothelial cells in-

ternalized fivefold more cell-bound [125 I]-insulin than pro-oxidant treated cells. The [125 I]-insulin disappeared from the cell surface at a similar rate in both pro-oxidant and untreated cells, with relatively more [125 I]-insulin being released into the medium in pro-oxidant treated cells. Although xanthine oxidase reduced insulin receptor mRNA and protein concentrations, cell surface insulin binding capacity was not affected. Following 5 min insulin exposure, insulin receptor auto-phosphorylation was considerably reduced in cells challenged with xanthine oxidase for 2 h, which could be important for insulin receptor activation and internalization.

Conclusion/interpretation. Oxidative stress impairs insulin endocytosis in both arterial and venous endothelial cell lines. This was not a consequence of modified insulin binding capacity but could involve insufficient insulin receptor activation. [Diabetologia (2001) 44: 605–613]

Keywords Insulin endocytosis, insulin receptor, oxidative stress, endothelial dysfunction, endothelial cells.

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Abbreviations: ROS, reactive oxygen species; IR, insulin receptor; BAEC, bovine aortic endothelial cells; XO, xanthine oxidase; HX, hypoxanthine; TCA, trichloroacetic acid.

Coexistence of insulin resistance and endothelial dysfunction is commonly observed in a variety of metabolic and cardiovascular disorders, including atherosclerosis and Type II (non-insulin dependent) diabetes mellitus [1]. Treatment of reduced insulin sensitivity with antioxidants, such as glutathione, vitamin C and vitamin E, can almost completely restore impaired endothelium-dependent vasorelaxation, a well established functional measure of endothelial dysfunction [2–4]. Because insulin sensitivity is susceptible to changes in whole-body redox-balance, oxidative stress has been increasingly asso-

ciated with the development of insulin resistance [5].

The endothelium can modify insulin sensitivity by regulating delivery of circulating glucose and insulin to peripheral tissues and actively transport these substances to the subendothelial space [6, 7]. The idea that the endothelium acts as a functional barrier to insulin is supported by several studies showing a substantial lag-time between insulin reaching peak concentrations in plasma and interstitial fluid, respectively [8, 9].

Recent reports indicate that insulin uptake and trafficking across the endothelium is mediated by the cell surface invaginations known as caveolae [10]. Treatment of endothelial cells with sterol-binding agents, which disrupt caveolae without altering the structural integrity of clathrin coated pits, significantly inhibit vesicular insulin processing [10]. Although caveolae could be a prerequisite for insulin transfer from the blood stream to the interstitial fluid, only a weak correlation exists between vesicle density and endothelial protein permeability [11]. Instead, differences in the expression of caveolae associated ligand-binding receptors involved in protein endocytosis have been proposed as an indicator of the exchange efficiency of macromolecules across the endothelium [11]. Evidence supporting the notion that transendothelial insulin processing is receptor-mediated is, firstly, unidirectional insulin transport is inhibited by anti-insulin receptor (IR) antibodies in vitro and in situ [12, 13]. Secondly, serine and tyrosine phosphorylation of the IR β -subunit has been shown to increase both internalization and externalization of insulin in vitro, suggesting a role for IR activation and phosphorylation in insulin transport [14]. Thirdly, deleting specific amino acid residues in the juxta-membrane domain of the IR or introducing point mutations affecting the intrinsic kinase activity, impedes considerably insulin internalization, possibly affecting insulin transcytosis [15, 16].

Several studies have associated oxidative stress with endothelial dysfunction and insulin resistance. For example, reduced concentrations of antioxidants, such as superoxide dismutase (SOD), vitamin C and reduced glutathione, have been documented in Type II diabetes [17]. Other findings demonstrate high concentrations of biological markers of oxidative stress in Type II diabetes, indicating an increased generation of reactive oxygen species (ROS) [18]. In addition, recent data shows that insulin resistant obese Zucker rats rapidly develop a Type II diabetes-like state with endothelial dysfunction when exposed to a pro-oxidative insult [19]. We therefore investigated the effect of the ROS generating system xanthine oxidase/hypoxanthine (XO/HX) on insulin uptake and processing in endothelial cells in vitro in order to determine if oxidative stress has a direct effect on insulin internalization.

Materials and methods

Materials. Enzymes and reagents for RT-PCR geneAmp were purchased from Perkin Elmer (Foster City, Calif., USA). The RNazol B was from Biogenesis (Poole, Dorset, UK). Human recombinant insulin was from Boehringer Mannheim (Lewes, East Sussex, UK). Protein molecular weight markers, nitrocellulose membranes, enhanced chemiluminescence substrates, sheep anti-mouse and donkey anti-rabbit IgG antibody and moniodinated human [Tyr^{A14}]-insulin ([¹²⁵I]-insulin; 74 TBq/mmol) were purchased from Amersham Life Science (Little Chalfont, Buckinghamshire, UK). Rabbit polyclonal anti-IR- β antibody and mouse monoclonal anti-phosphotyrosine antibody was from Autogen Bioclear (Caine, Wiltshire, UK).

Cell culture. Ea.Hy926 (human umbilical vein endothelial cells fused with a human pulmonary epithelial cell line (A549)) [20], and bovine aortic endothelial cells (BAEC) were cultured in DMEM containing 5 mmol/l D-glucose, 4 mmol/l L-glutamine and 10% fetal calf serum at 37°C in humidified air/CO₂ (95%/5%). Before adding stimulant, confluent cells were deprived of serum for 18 to 22 h. All experiments were subsequently carried out in serum-free DMEM. The Ea.Hy926 cells was a kind gift from Dr C.-J. S. Edgell (Department of Pathology, University of North Carolina, USA).

Insulin binding and internalization. Assessment of insulin binding and internalization was done as described previously [15]. Briefly, cells were incubated at 4°C for 18 to 20 h in B-buffer (100 mmol/l HEPES, 120 mmol/l NaCl, 1.2 mmol/l MgSO₄, 5 mmol/l KCl, 10 mmol/l glucose, 1 mmol/l EDTA, 15 mmol/l Na-acetate, 1% BSA, pH 7.8) containing 1.85 KBq/ml [¹²⁵I]-insulin (74 TBq/mmol) with or without unlabeled human insulin (10 μ mol/l). Cells were lysed by 1 mol/l NaOH and radioactivity was measured. Part of the cell lysate was used to determine total cellular protein concentrations (Bio-Rad, Hemel Hemstead, Hertfordshire, UK). Specific binding (cpm ng cell protein⁻¹ml⁻¹) was calculated by subtracting non-specific binding from total binding.

In order to measure the degree of insulin internalization and degradation, cells were exposed to [¹²⁵I]-insulin for 18 to 20 h at 4°C followed by incubation with 1 ml of pre-warmed B-buffer at 37°C for varying times (5, 15, 30, 60 min) as described previously [15]. Further internalization was stopped by transferring the culture plates onto ice. An aliquot of the medium was taken and trichloroacetic acid (TCA)-precipitable and TCA-soluble radioactivity measured. Cell surface associated [¹²⁵I]-insulin was removed by incubating the cells briefly in washing buffer (0.2 mol/l acetic acid, 0.5 mol/l NaCl, pH 3.0). The washing medium was subsequently assessed for radioactivity. Finally, cells were solubilized in 1 mol/l NaOH for 2 h (4°C) and cell associated radioactivity quantitated as a measure of internalized [¹²⁵I]-insulin.

RT-PCR. Following cell treatment, RNA was isolated according to the RNazolB method. The IR mRNA concentrations were detected with specific primers by RT-PCR using a geneAmp RNA PCR kit according to instructions supplied. The PCR products were separated by 1% agarose gel electrophoresis and stained by ethidium bromide. PCR products were quantified by scanning densitometry using the Biorad Gel-doc system and expressed as a percentage compared to untreated cells.

Immunoprecipitation and immunoblotting. Cell lysates were incubated overnight with a rabbit IR- β polyclonal antibody (fi-

Fig. 1A–D. The XO/HX impairs [125 I]-insulin endocytosis in BAEC. [125 I]-insulin was incubated with untreated BAEC (●) or BAEC exposed to 10 mU/ml XO; 1 mmol/l HX for 2 h (○), in the absence or presence of 10 μ mol/l insulin for 18 to 20 h (4°C). Unbound [125 I]-insulin was washed away and [125 I]-insulin processing assessed at 37°C at the designated time-points. Cells were washed with acetic acid to remove cell surface bound [125 I]-insulin (**A**) and solubilized to calculate intracellular [125 I]-insulin concentrations (**B**). The medium was removed and TCA-precipitable and TCA-soluble radioactivity measured as an estimate of intact [125 I]-insulin (**C**) and degraded [125 I]-insulin (**D**). Results are expressed as percentage of specific membrane-bound radioactivity at $t = 0$. Data are representative of three independent experiments.

nal concentration: 1 μ g/ml). The immunocomplexes were then coupled to ProteinG Sepharose (1:1 v/v) during a 4-h incubation period. After separation of the immunoprecipitates by SDS-PAGE, proteins were immunoblotted with a rabbit IR- β polyclonal antibody against the 95 K IR- β -subunit (final concentration: 1 μ g/ml) or a mouse monoclonal anti-phosphotyrosine antibody (final concentration: 2 μ g/ml). Blots were exposed to donkey anti-rabbit IgG antibody (1/5000) or sheep anti-mouse IgG antibody (1/1250) and immunoreactive proteins detected using enhanced chemiluminescence.

Statistical analysis. Data are given as means $\pm$ SEM of at least three independent observations. Statistical analysis was done by one-sample t-test or by unpaired Student's t test when comparing treatments at specific time points. A p value of less than 0.05 was considered to be statistically significant.

Results

The effect of oxidative stress on insulin uptake in endothelial cells. Following a 2-h exposure to 10 mU/

ml XO; 1 mmol/l HX (these experimental conditions did not affect cell viability), BAEC were equilibrated at 4°C with [Tyr^{A14}]-insulin ([125 I]-insulin), which displays binding characteristics and a biological potency similar to native insulin [21]. To investigate the temporal pattern of cell associated [125 I]-insulin processing, cells were subsequently incubated at 37°C for 5 to 60 min.

The [125 I]-insulin disappeared from the cell surface at essentially the same rate in both control and XO/HX treated BAEC, declining to 25–35 % of its initial value after 15 min incubation (Fig. 1A). Part of the dissociated [125 I]-insulin was released into the medium (Fig. 1C), while a smaller amount was internalized (Fig. 1B). Of note, control cells were shown to endocytose [125 I]-insulin more rapidly and to a greater extent than cells exposed to XO/HX. Thus, after 15 min, more than 25 % of cell-bound [125 I]-insulin was internalized in control cells, while XO/HX treated cells only endocytosed about 5 % of total cell associated [125 I]-insulin. In contrast, XO/HX clearly promoted the release of TCA-precipitable [125 I]-insulin into the medium, although this was not statistically significant (Fig. 1C). The [125 I]-insulin degradation, as determined by an increase in non-TCA-precipitable radioactivity, was most pronounced in control cells, reaching about 7 % of total cell associated [125 I]-insulin by 60 min, while degraded amounts of [125 I]-insulin remained stable at approximately 2 % over the entire incubation period in BAEC incubated with XO (Fig. 1D).

A similar profile on insulin internalization was observed in Ea.Hy926 cells challenged with 20 mU/ml XO; 1 mmol/l HX (data not shown). Although the endocytotic response to XO was comparable, we noted a 50 % reduction in total [125 I]-insulin binding ca-

Fig. 2A, B. [¹²⁵I]-insulin internalization efficiency differs between Ea.Hy926 and BAEC. The [¹²⁵I]-insulin was allowed to bind in the absence or presence of 10 μmol/l insulin for 18–20 h at 4 °C. Unbound [¹²⁵I]-insulin was washed away and cell associated radioactivity measured (A) or [¹²⁵I]-insulin internalization assessed at 37 °C at the designated time-points in BAEC (○) and Ea.Hy926 cells (●) (B). Cells were washed with acetic acid to remove cell surface bound [¹²⁵I]-insulin and solubilized to calculate intracellular [¹²⁵I]-insulin levels. Results are expressed as percentage of specific membrane-bound radioactivity at t = 0. All data are representative of three to five independent experiments. Comparison of [¹²⁵I]-insulin binding capacity between BAEC and Ea.Hy926 cells was done using an unpaired Student's *t* test (* *p* < 0.05)

capacity in untreated Ea.Hy926 cells compared with BAEC (Fig. 2A). This difference was further supported by the greater extent of [¹²⁵I]-insulin uptake in BAEC relative to Ea.Hy926 cells (Fig. 2B).

Characterization of insulin uptake in endothelial cell preparations. Receptor-mediated insulin uptake and subsequent transcytosis has been reported in several macrovascular and microvascular endothelial cell lines including BAEC [22]. Because an inherent variability between different BAEC preparations has, however, been documented [23], it was necessary to examine whether anti-IR antibodies could diminish [¹²⁵I]-insulin binding in vitro in our cells.

In accordance with previous studies we observed a concentration dependent decrease in [¹²⁵I]-insulin cell association when incubating the cells with anti-IR antibodies (Fig. 3). It has previously been described that, when using transfected NIH-3T3 cells, a brief acetic acid wash (pH 3) is sufficient to extract more than 90 % of cell surface associated [¹²⁵I]-insulin at 4 °C [15]. Likewise, we observed that less than

Fig. 3. Anti-IR-α-antibodies inhibit binding of [¹²⁵I]-insulin to BAEC in a concentration dependent manner. [¹²⁵I]-insulin was allowed to bind to BAEC in the presence of varying concentrations of anti-IR-α-antibodies for 18 to 20 h at 4 °C. Unbound [¹²⁵I]-insulin was washed away and cell associated radioactivity measured. Results are expressed as percentage of membrane-bound radioactivity in the absence of antibody. Data are representative of three independent experiments

10 % of pre-bound [¹²⁵I]-insulin remained cell associated following a similar procedure with BAEC, suggesting that insulin internalization is an active and energy-dependent process.

Endothelial cells have previously been shown to contain an abundant population of caveolae, which participate in transcytosis of proteins such as albumin and insulin both in vitro and in situ [10, 24]. Exposing endothelial cells to the sterol-binding agent filipin, disrupts the structural integrity of caveolae resulting in rapid inhibition of insulin transport [10]. Although a recent report concludes that clathrin-coated pathways are altered after acute cholesterol depletion using β-methyl-cyclodextrin [25], both morphological and functional studies have shown that filipin specifically disrupts caveolae [10, 26].

A 15 min filipin treatment of BAEC resulted in a concentration dependent impairment in initial [¹²⁵I]-insulin uptake (Fig. 4A). This was not the result of alterations in endothelial [¹²⁵I]-insulin binding capacity, because [¹²⁵I]-insulin specific binding was not affected by filipin (Fig. 4B). The non-sterol-binding agent xylazine was used as a control to ensure that insertion of lipid-binding components into the cell membrane, which could have a potentially detrimental effect on lipid bilayer arrangement and IR organization, was not responsible for abnormal insulin uptake [10]. Xylazine had no effect on [¹²⁵I]-insulin binding capacity or [¹²⁵I]-insulin internalization (Fig. 4).

Total insulin cell association in response to XO/HX treatment. We wanted to investigate if XO/HX induced reductions in [¹²⁵I]-insulin endocytosis were a consequence of alterations in IR expression or of abnormal IR activation.

Fig. 4 A, B. Filipin III impairs $[^{125}\text{I}]$ -insulin internalization without affecting $[^{125}\text{I}]$ -insulin binding capacity in BAEC. BAEC, exposed to varying concentrations of filipin III or xylazine for 15 min, were equilibrated with $[^{125}\text{I}]$ -insulin in the absence or presence of 10 $\mu\text{mol/l}$ insulin for 18–20 h at 4 °C. **A**, Unbound $[^{125}\text{I}]$ -insulin was washed away and $[^{125}\text{I}]$ -insulin internalization calculated in filipin III (●) or xylazine treated (○) BAEC after 15 min incubation at 37 °C. Before solubilization cells were washed with acetic acid to remove cell surface bound $[^{125}\text{I}]$ -insulin. **B**, Unbound $[^{125}\text{I}]$ -insulin was washed away and cell associated radioactivity measured in filipin III (■) or xylazine treated (□) BAEC as a measure of $[^{125}\text{I}]$ -insulin binding capacity. All values are expressed as percentage of untreated cells. Data are representative of three to four independent experiments

When Ea.Hy926 cells were exposed to 20 mU/ml XO; 1 mmol/l HX, IR mRNA concentrations were reduced to $56 \pm 5\%$ and $49 \pm 6\%$ of control levels after 1-h and 2-h incubation, respectively ($p < 0.05$; $n = 3$; Fig. 5). Concomitant catalase treatment (160 U/ml) reversed the IR mRNA down regulation to $150 \pm 30\%$ ($p = 0.089$; $n = 3$) and $117 \pm 11\%$ ($p < 0.01$; $n = 3$) of control values after either 1-h or 2-h incubation, indicating the potential of H_2O_2 or H_2O_2 -derived molecules to induce significant reductions in IR mRNA concentrations. Western blot analysis of samples immunoprecipitated with an anti-IR- β antibody showed a $54 \pm 20\%$ decrease in IR protein concentrations relative to control cell following 2-h of XO/HX incubation (Fig. 6).

Although these findings initially suggest an association between the size of the cellular IR pool and insulin endocytosis efficiency, this association was not reflected in any XO/HX induced alterations in $[^{125}\text{I}]$ -insulin binding characteristics (Fig. 7), suggesting that the probable effect of ROS occurs after insulin/IR interaction.

Fig. 5 A, B. Ea.Hy926 cells were exposed to either 20 mU/ml XO; 1 mM HX or XO/HX in combination with 160 U/ml catalase for the designated time. Total RNA was isolated and RT-PCR carried out with primers specific for IR and GAPDH. After electrophoresis in a 1% agarose gel, PCR products were stained by ethidium bromide (**A**) and quantified by scanning densitometry (**B**). Filled bars represent exposure to XO/HX and open bars denote incubation with both XO/HX and catalase. All data are representative of three independent experiments and expressed as percentage of untreated cells. * $p < 0.05$ between untreated and XO/HX treated cells using one-sample t-test. $\psi p < 0.01$ comparing specific timepoints using unpaired Student's *t* test

Because the efficiency of insulin uptake could be susceptible to changes in the phosphorylation state of the IR [14], we examined if XO/HX alters insulin stimulated tyrosine phosphorylation of the IR- β -subunit. Before insulin stimulation, IR tyrosine phosphorylation was not detectable (data not shown). Following 5-min insulin exposure (100 nmol/l), IR tyrosine phosphorylation was reduced to $43 \pm 17\%$ of control levels in Ea.Hy926 exposed to XO/HX for 2 h ($p < 0.05$; $n = 4$; Fig. 8).

Discussion

The endothelium synthesizes and is exposed to low concentrations of ROS, some of which participate in intracellular signalling and redox-sensitive gene regulation [27]. Where endothelial dysfunction is associated with diseases such as Type II diabetes, oxidative stress is evident possibly because of a combination of

Fig. 6 A, B. Immunoprecipitates from untreated Ea.Hy926 cells (■) and Ea.Hy926 cells exposed to 20 mU/ml XO; 1 mmol/l HX for 2 h (□) were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with an anti-IR- β antibody (A). Relative IR protein concentrations were quantified by scanning densitometry and expressed as percentage of untreated cells (B). Data are representative of four independent experiments

increased ROS generation and a reduced efficiency of endogenous antioxidant defense systems [17, 28]. In conditions associated with insulin resistance and high concentrations of ROS, a scenario of blunted endothelial cell responses are frequently observed [1, 29]. It remains to be established however whether alterations in the physical and physiological integrity of the endothelium can also affect the ability of the endothelium to facilitate the processing of different macromolecules across the vessel wall, particularly insulin.

Our data show that a pro-oxidative insult is able to specifically reduce receptor-mediated insulin internalization in venous and arterial endothelial cells *in vitro*. This cannot be explained simply by a change in IR number but could reflect an effect on insulin signal transduction, either directly or indirectly, which in turn modulates insulin internalization. Surprisingly little is known about the signalling pathways responsible for endothelial insulin transcytosis. In contrast to classical insulin sensitive cells where internalization of the insulin/IR complex results in complete degradation of the ligand, insulin degradation pathways seem to be of only minor importance in endothelial cells [22], suggesting the existence of endo-

Fig. 7. XO/HX has no effect on [¹²⁵I]-insulin binding capacity in BAEC. [¹²⁵I]-insulin was allowed to bind to untreated BAEC (■) or BAEC exposed to 10 mU/ml XO; 1 mmol/l HX (□) for 2 h, in the absence or presence of 10 μ mol/l insulin for 18 to 20 h (4 °C). Unbound [¹²⁵I]-insulin was washed away and cell associated radioactivity measured. Data are representative of four independent experiments

thelial specific signalling cascades. In addition, some degree of variation between endothelial cells derived from different vascular beds seems to exist, with least insulin degradation occurring in capillary endothelium [22]. In combination with higher insulin binding capacity these features could emphasize a possibly greater physiological relevance of insulin delivery across capillaries supplying e.g. skeletal muscle and adipose tissue. It has been suggested that defective IR kinase activity is related to insulin resistance *in vivo* and is associated with impaired insulin internalization *in vitro*, indicating the significance of IR activation [15]. Furthermore, the coincidence of IR tyrosine phosphorylation and insulin endocytosis indicates a requirement for IR auto-phosphorylation in insulin uptake [14]. We showed that XO/HX reduces IR auto-phosphorylation possibly resulting in insufficient IR activation and reduced insulin uptake. Although alterations in IR stimulation are paralleled by significant reductions in IR mRNA and a distinct decrease in IR protein amounts, we report that insulin binding capacity is not altered. This indicates that endothelial cells maintain a constant level of membrane associated IR. Indeed, studies have shown that the number of membrane associated IR in the endothelium is regulated, i.e. a considerable intracellular IR reserve could exist with the potential to control and replenish the amount of IR at the cell surface [14]. Therefore, if ROS mediated effects on IR expression can be circumvented by increased IR recruitment to the cell membrane, insulin/IR interaction and subsequent IR auto-phosphorylation would not be expected to change. This interpretation would

Fig. 8 A, B. Insulin stimulated IR tyrosine phosphorylation is decreased in Ea.Hy926 cell preparations after XO/HX exposure. Ea.Hy926 cells were incubated with 20 mU/ml XO; 1 mmol/l HX for 1 h or 2 h and subsequently exposed to 100 nmol/l insulin for 5 min. Cell lysates were immunoprecipitated with an anti-IR- β antibody and separated by SDS-polyacrylamide gel electrophoresis. The blots were probed with an anti-phosphotyrosine antibody and visualized by enhanced chemiluminescence (**A**) and quantitated by scanning densitometry (**B**). Data are representative of four independent experiments and are expressed as percentage of untreated cells. * $p < 0.05$ relative to corresponding control value using one-sample t test

favour down regulation of IR auto-phosphorylation as a contributing factor of XO/HX-impaired insulin internalization. It is noteworthy that normal IR cell surface distribution has been reported to be associated with decreased IR auto-phosphorylation and insulin endocytosis in hepatocytes isolated from STZ-induced diabetic rats, noted to be prone to oxidative stress [30]. In addition, studies on fibroblasts and adipocytes have demonstrated that H_2O_2 is a potent inhibitor of insulin signalling pathways and significantly impairs insulin responsiveness [31].

At the endothelial cell surface, IR are primarily localized in caveolae along with other proteins involved in classical insulin signal transduction, emphasizing the caveolae as a focal point for cellular insulin signalling [32]. We demonstrate that insulin uptake in BAEC is likely to be caveolae dependent, based on the observation that filipin impairs insulin internalization. It is interesting to note that a recent report has suggested a role for XO derived ROS in caveolae disruption in BAEC because of membrane dissociation of the regulatory component caveolin [24]. Because caveolin augments insulin-stimulated IR kinase activity, possibly by stabilizing the activated conformation of the tyrosine phosphorylated IR, an effect of XO on IR activation cannot be excluded [33].

There is evidence for a restrictive nature of insulin processing from plasma to subendothelial insulin targets. For example, transendothelial insulin delivery has been proposed to represent a rate-limiting step in the onset of insulin action because of a markedly delayed response in insulin kinetics in the interstitial fluid compared with plasma [8, 9]. This could explain why the glucose disposal rate is closely correlated with insulin kinetics in the interstitial fluid but only loosely with changes in insulin plasma concentrations [34]. Moreover, transfer of low affinity insulin analogues from the blood stream to muscle tissue in situ is significantly impaired as is insulin processing in the presence of anti-IR antibodies against endothelial IR [13]. Further indications of a receptor-mediated insulin passage across the endothelium have been provided by human studies, where two distinctly different methods have revealed a saturating effect on insulin transport into the interstitial space when increasing infused insulin concentrations [8, 35]. In contrast, other reports argue against a receptor-mediated and hence saturable transport mechanism, instead predicting a diffusory or a bidirectional convective transport pathway [36, 37]. Thus, the mechanisms responsible for impairments in transendothelial insulin transport [38, 39] could involve altered capillary permeability, abnormal receptor facilitated insulin delivery or reduced insulin stimulated tissue perfusion. Insulin's vasorelaxing effect, which has been said to increase skeletal muscle blood flow and thereby facilitate glucose disposal, is, for example, diminished in Type II diabetes [40, 41]. Because the vascular response to insulin is likely to be nitric oxide (NO) dependent and involves IR kinase activity [42], it would be of interest to investigate whether the observed effect of ROS on IR tyrosine phosphorylation could directly affect endothelial NO production.

The relevance of receptor-mediated insulin processing remains unclear and no direct evidence of an effect of ROS on transendothelial insulin delivery in vivo has been documented in this study. Nonetheless, it is tempting to speculate that oxidative stress could be associated with reduced plasma insulin clearance, possibly resulting in impaired peripheral insulin utilization. It is important, however, to consider the association between oxidative stress and increments in endothelial permeability. Increased protein permeability is apparent, for example, in animal models associated with oxidative stress and insulin resistance [43, 44]. Thus, increased accessibility of macromolecules such as insulin to the subendothelial compartment could "compensate" for reduced transendothelial transport. Further work is required in order to determine if endothelial permeability and insulin transcytosis are equally sensitive to increased free radical activity.

In conclusion, we have shown that oxidative stress generated by XO/HX is able to specifically reduce re-

ceptor-mediated insulin endocytosis in two different endothelial cell lines. This was not due to alterations in insulin binding capacity but could reflect a change in IR auto-phosphorylation possibly affecting the intrinsic capacity of insulin to stimulate its own uptake. Because endothelial IR could have a role in insulin transcytosis, it remains to be seen whether ROS also modifies insulin processing across the capillary wall in insulin sensitive tissue.

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