The Mechanism of Oxidative Stress Stabilization of the Thromboxane Receptor in COS-7 Cells*

Received for publication, June 25, 2003, and in revised form, October 16, 2003
Published, JBC Papers in Press, October 28, 2003, DOI 10.1074/jbc.M306761200

Francois Valentin‡§, Mark C. Field‡§, and John R. Tippins‡|

From the ‡Department of Biological Sciences and the §Welcome Trust Laboratories for Molecular Parasitology, Imperial College, Exhibition Road, London SW7 2AZ, United Kingdom

The 8-iso-prostaglandin F2α, a prostanoid produced in vivo by cyclooxygenase-independent free-radical-catalyzed lipid peroxidation, acts as a partial agonist on the thromboxane receptor (TXA2R) and is a potent vasoconstrictor in the oxidatively stressed isolated perfused rat heart. We hypothesized that response in the isolated heart may be due to augmentation of TXA2R density, which may be initiated by the presence of oxidative radicals. Previous studies have shown that TXA2R density is increased during atherosclerosis on both the medial and intimal smooth muscle layers in human coronary arteries. Here we describe the effect of oxidative stress on TXA2R. The thromboxane A2 receptor β isoform (TXA2Rβ) was transiently expressed in COS-7 cells. Immunofluorescence suggested that the presence of H2O2 increased translocation of TXA2Rβ from the endoplasmic reticulum (ER) to the Golgi complex. H2O2 treatment also increased binding of a TXA2R antagonist ([3H]SQ29548) to membranes. Degradation kinetics of TXA2Rβ following cycloheximide treatment, a protein synthesis inhibitor, suggested not only that TXA2Rβ is a short-lived protein predominantly localized to the ER but also that TXA2Rβ degradation is modulated in the presence of H2O2. Our results indicate that oxidative stress induces maturation and stabilization of the TXA2Rβ protein probably by intracellular translocation. Importantly, these observations also suggest that TXA2Rβ levels are modulated by ER-associated degradation and controlled by the efficiency of transport to post-ER compartments. Stabilization of the TXA2Rβ by translocation from a degradative compartment, i.e. the ER, can account for the augmentation of receptor density observed in vivo.

Thromboxane A2 (TXA2) is an unstable arachidonate metabolite, implicated as a mediator in diseases such as myocardial infarction, stroke, and bronchial asthma (1). Binding of TXA2 to its receptor, a polytopic membrane-spanning protein, induces vasoconstriction and platelet aggregation, as well as mitogenesis and hypertrophy of vascular smooth muscle cells (2). Two TXA2 receptor (TXA2R) isoforms have been identified, TXA2Rα (343 amino acids), which is mainly located in the placenta, and TXA2Rβ (407 amino acids), located in the endothelium; these isoforms are generated by the alternative splicing of a single gene (3, 4). The TXA2R is part of the G protein-coupled receptor superfamily, and evidence suggests that TXA2R-induced production of second messenger inositol polyphosphates results from the activation of the Gq11 family of heterotrimeric G proteins (5).

Isoprostanes are formed by free radical attack on membrane phospholipids during oxidative stress (6). They are found in increased concentration in patients with coronary heart disease and are potent vasoconstrictors (7). We have shown that one of these, the 8-iso-prostaglandin F2α, is a potent coronary vasoconstrictor, and its effect is exerted via partial agonist action on the TXA2R (8). This mechanism of action on TXA2R, in vascular smooth muscle and in platelets, has been confirmed in a TXA2R knock-out mouse (9). Our data suggested that a critical determinant of the intrinsic activity of the isoprostane is the TXA2R reserve, and this has subsequently been supported by another study (10). We have shown that, in the normal rat heart perfused at constant pressure in the Langendorf mode, 8-iso-prostaglandin F2α had no effect, even though U46619, a TXA2R agonist, produced a pronounced vasoconstriction. However, after an oxidative stress induced by 30 min of low flow and reperfusion or by a superoxide-generating system (i.e. xanthine and xanthine oxidase), 8-iso-prostaglandin F2α became a potent vasoconstrictor, whereas the response to U46619 was unchanged (11). Responses to both agonists were inhibited by the TXA2R antagonist SQ29548, suggesting that they act upon the same receptor.

The rapidity of the change in response suggests that this is unlikely to be due to alterations in gene expression and could be explained in several ways including loss of NO-mediated physiological antagonism (12), changes in the second messenger systems that transduce the response to isoprostane, or an increase in receptor reserve. Evidence for this last possibility has been gained from clinical studies: platelet TXA2R expression is increased in patients in acute myocardial infarction (13), and in human coronary arteries TXA2R density is augmented during cardiovascular disease (14, 15). One of the possible mechanisms for oxidative stress to increase the TXA2R reserve could be the relocation of an inactive pool of receptor; in hypoxic or ischemic hearts, oxidative stress may induce the translocation of glucose transporters GLUT 1 and GLUT 4 (16). Alternatively, stabilization of the protein could account for activation; in melanoma cells, dihydroxyphenylalanine induces the transition of misfolded wild type tyrosinase to the native and functional form, which is competent to exit the ER (17). We hypothesized that oxidative stress could act on the TXA2R folding, as dihydroxyphenylalanine on tyrosinase, through the

*This work was supported by British Heart Foundation Grant PG/2000119 (to J. R. T. and M. C. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence may be addressed. Tel./Fax: 44-020-7594-5277; E-mail: m.field@imperial.ac.uk
¶ To whom correspondence may be addressed. Tel.: 44-020-7594-5216; Fax: 44-020-7594-5300; E-mail: j.tippins@imperial.ac.uk
§ The abbreviations used are: TXA2, thromboxane A2; TXA2R, thromboxane A2 receptor β isoform; ER, endoplasmic reticulum; BFA, brefeldin A; UPR, unfolded protein response; HCASMc, human coronary artery smooth muscle cells; CHO, Chinese hamster ovary; DAPI, 4’,6-diamidino-2-phenylindole; GFP, green fluorescent protein; DTT, dithiothreitol.
unfolded protein response (UPR) mechanism, which is activated under a variety of stress conditions (18). In this present study, we expressed the TXA2Rβ isoform in COS-7 cells by transient DNA transfection, and we have investigated the effect of exposure to hydrogen peroxide on the post-translational behavior of the TXA2Rβ isoform.

**EXPERIMENTAL PROCEDURES**

**Materials**—Simian kidney (COS-7) cells were obtained from the American Type Culture Collection. Human coronary artery smooth muscle cells (HCASMc) were obtained from CellWorks (Buckingham, UK). The complete control inducible mammalian expression system (pERV3 CHO stable cell line and the pEGSH vector) was purchased from Stratagene (La Jolla, CA). TXA2Rβ cDNA was kindly provided by Drs. J. A. Ware and A. W. Ashton (Albert Einstein College of Medicine, New York). Mammalian expression vector pcDNA 3.1/CT-GFP-TOPO, pcDNA 4/CT-Myc-His, Dulbecco’s modified Eagle’s medium supplemented with glucose (4.5 g/liter), fetal bovine serum, and antibiotic/antimycotic solution were purchased from Invitrogen. 4’,6-diamidino-2-phenylindole (DAPI), anti-Golgin-97 mouse monoclonal antibody, antimycotic solution were purchased from Invitrogen. 4’,6-diamidino-2-phenylindole (DAPI) from Sigma Chemical Co. (Ann Arbor, MI). DNA Taq polymerase was obtained from Stratagene. The Bradford protein assay kit was from Bio-Rad. Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG, horseradish peroxidase-conjugated rabbit anti-goat IgG, anti-Myc Cy3, or fluorescein isothiocyanate-conjugated mouse monoclonal antibody, and all of the other chemicals were purchased from Sigma.

**Subcloning of C-terminal GFP and Myc-Tagged Human TXA2Rβ cDNA**—Human TXA2Rβ cDNA was amplified by PCR. The oligonucleotides used were 5’-CGGGATCCATGTGGCCCAACGGCAGT-3’ and 5’-CGGGATCCATGTGGCCCAACGGCAGT-3’ and 5’-GGATCCCGCTGCTGATC-3’ with BamHI and EcoRV sites (underlined). The PCR product was subcloned into pcDNA 3.1/CT-GFP-TOPO or into the pcDNA 4/CT-Myc-His at the BamHI and EcoRV sites. The insertion of the TXA2Rβ cDNA was confirmed by DNA sequencing. The human TXA2Rβ wild type vector was made by PCR using the following primers: 5’-CGGGATCCATGTGGCCCAACGGCAGT-3’ and 5’-GGATCCCGCTGCTGATC-3’ with BamHI and an introduced stop codon (underlined). The PCR product was subcloned into the mammalian expression vector pcDNA 3.1/CT-GFP-TOPO and verified by sequencing.

**Subcloning of C-terminal Myc-Tagged Human TXA2Rβ cDNA into pEGSH Vector**—Human C-terminal Myc-Tagged human TXA2Rβ cDNA was amplified by PCR from the pcDNA 4/CT-Myc-His construct (see above). The oligonucleotides used were 5’-GGATCCCTATGGGCCCACGGCAGT-3’ and 5’-GGATCCCGCTGCTGATC-3’ with EcoRV sites (underlined). The PCR product was subcloned into pEGSH vector at EcoRV site. DNA sequencing and DNA digestion confirmed the insertion and the orientation of the TXA2Rβ-Myc-cDNA.

**Cell Culture and Expression of the GFP and Myc-Tagged Human TXA2Rβ**—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotic-antimycotic at 37 °C in a humidified atmosphere of 95% air and 5% CO2. pERV3 CHO stable cell lines were maintained in presence of G418 (500 µg/ml), and HCASMc were maintained in HCASMc basal medium supplemented with HCASMc growth supplement (CellWorks). To create cell lines expressing TXA2Rβ, pcDNA 3.1/CT-GFP-TOPO, or pcDNA 4/CT-Myc-His expression vector containing the cDNAs of the wild type TXA2Rβ, GFP-tagged TXA2Rβ (pcDNA3.1/TXA2Rβ-GFP), or Myc-Tagged TXA2Rβ (pcDNA4/ TXA2Rβ-Myc-His) were transfected into the cells using FuGENE 6 transfection reagent (Roche Applied Science). To obtain the COS-7 stable cell lines, COS-7 cells were transfected with pcDNA 4/CT-Myc-His expression vector and maintained in Dulbecco’s modified Eagle’s medium in the presence of zeocin (600 µg/ml) for 8 weeks.

**Antibody Production**—For antibody production, residues 270–369 of
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TXA₂Rβ was amplified using DNA Taq polymerase with the following primers: 5'-CCGAGATCCCGAAGACCCGCTGCA-3' and 5'-GGCATGGAATTGGCGCTGTAATCC-3', cloned into the expression vector pEX-2TK (Amersham Biosciences) through BamHI and EcoRI sites (undertiled), and expressed as a glutathione S-transferase fusion protein in Escherichia coli DH5α. Glutathione S-transferase fusion protein was inoculated into rabbits with Freund's incomplete adjuvant (Sigma), a procedure repeated four times. Specific antibodies were affinity-purified on antigen immobilized on cyanogen bromide-activated Sepharose 4B (Amersham Biosciences).

Subcellular Localization of TXA₂Rβ—COS-7 cells were transfected using pCDNA4/TXA₂Rβ-Myc-His. At 24 h post-transfection, COS-7 cells were treated with cycloheximide (200 μg/ml, 2 h) by adding the drug to the medium, and then the cells were submitted to oxidative stress (H₂O₂, 10 μM, 40 min). The cells were washed twice in 10 mM triethanolamine, 10 mM acetic acid, 250 mM sucrose, 1 mM EDTA, and 1 mM dithiothreitol, harvested with a rubber policeman in 800 μl of buffer containing protease inhibitors (protease inhibitor mixture tablets; Roche Applied Science), and homogenized by 15 passages through a 25-gauge needle on a 1-ml syringe. Nuclei and intact cells were removed by microcentrifugation at 1200 x g for 5 min at 4 °C. The postnuclear supernatant was loaded on preformed Nycodenz gradients prepared exactly as described (19). The postnuclear supernatant was loaded on top of the gradients and centrifuged for 1.5 h at 46,000 rpm in a Beckman L5–55 ultracentrifuge. Equal fractions were collected, and aliquots of each fraction were subjected to electrophoresis on SDS-PAGE gels. The distribution of TXA₂Rβ, calnexin (ER marker), and β-COP (intermediate compartment/Golgi complex marker) in the gradients was determined by immunoblotting.

Immunoblotting—Protein estimation was performed (Bradford assay), and 20 μg of cell lysate protein/lane were electrophoresed on 12% SDS-polyacrylamide gels and blotted onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences) by wet transfer. The filters were blocked in 5% milk, phosphate-buffered saline, and 0.1% Tween 20, probed with primary antibodies (in a 10-ml block) for 1 h, washed four times with phosphate-buffered saline/Tween 20, and then incubated with secondary antibody (diluted 1:5000) in blocking buffer for 1 h and washed. Detection was by chemiluminescence.

Kinetic Analysis—COS-7 cells were grown on coverslips and transiently transfected with pCDNA4/TXA₂Rβ-Myc-His as described above. At 24 h post-transfection, the COS-7 cells were treated with cycloheximide (200 μg/ml, 2 h) by adding the drug to the medium, and then cells were submitted to oxidative stress (H₂O₂, 10 μM, 40 min). The medium was replaced by a new medium containing cyloheximide throughout the experiment. At the indicated time the cells were collected and subjected to electrophoresis on SDS-PAGE gels. Loading control was performed using an anti-tubulin mouse antibody (gift from Keith Gull, Oxford, UK).

Binding Analysis—COS-7 cells were grown on coverslips and transiently transfected with pCDNA4/TXA₂Rβ-Myc-His as described above.
At 24 h post-transfection, the COS-7 cells (10^6) were homogenized in 1 ml of binding buffer (25 mM Tris-HCl, pH 7.4, 5 mM CaCl_2, 10 μM indomethacin, 50 μg/ml glutathione) using a cell homogenizer. After 5 min of centrifugation at 10,000 × g, the resulting supernatant was centrifuged at 150,000 × g for 30 min at 4 °C. The pellet corresponding to the membrane fraction was further resuspended in 800 μl of binding buffer and homogenized. For the binding assay, 50 μg of protein of the membrane fraction was incubated with a TXA2R antagonist ([^3H]SQ29548, 30 Ci/mol, 100 nM; PerkinElmer Life Sciences) in the presence or absence of 5 μM of unlabeled (cold) SQ29548 in a 0.1-ml reaction volume with vigorous shaking at room temperature for 60 min. The reaction was then terminated by adding 1 ml of ice-cold washing buffer (25 mM Tris-HCl, pH 7.4). The unbound ligand was filtered under vacuum through a Whatman GF/C glass filter (Whatman, Clifton, NJ) presoaked with the ice-cold washing buffer. The radioactivity of the TP and finally polymerized at 60 °C for 1 h. After rinsing in buffer, the cells were then dehydrated in an ethanol series, adding 1% uranyl acetate at the 30% stage, followed by propylene oxide and then embedded in Epon/Arnaldite 502 and finally polymerized at 60 °C for 48 h. The sections were cut on a Leica Ultracut T ultramicrotome at 70 nm using a diamond knife, contrasted with uranyl acetate and lead citrate, and examined on a Philips CM100 transmission electron microscope.

**RESULTS**

**Detection and Localization of TXA2Rβ in COS-7 Cells**—COS-7 cells express an endogenous TXA2Rα (20), suggesting that these cells have the intracellular signaling pathway with which to transduce a response to isoprostane and therefore constitute a relevant model system for this study. Determination of expression of Myc- His-tagged TXA2Rβ in COS-7 cells was performed by Western blotting (Fig. 1A). No detectable level of endogenous TXA2Rβ-Myc-His was observed in the non-transfected cell lysate (Fig. 1A, lane 1). In contrast, monoclonal antibody was able to detect TXA2Rβ-Myc-His in the transiently transfection, the cells were incubated 3 h at 37 °C in the presence of 200 μg/ml of cycloheximide (control), submitted to oxidative stress, fixed, permeabilized, and stained with the anti-golgin-97 monoclonal antibody and anti-mouse Texas Red-conjugated antibody as described above. The slides were examined using a confocal laser scanning microscope (Axioplan 2 with LSM 510; Carl Zeiss Inc.) equipped with 100×/1.4 Plan-ACROMAT oil immersion objective. GFP and Texas Red were excited with 488 and 595 nm of krypton-argon lasers, respectively, and individual channels were scanned in series to prevent cross-channel bleed through. Each image represents a single 0.4 μm “z” optical section of GFP-transfected cells.
transfected cell lysate (Fig. 1A, lane 2). Interestingly, the addition of H2O2 (10 μM, 40 min) increased the quantity of TXA2R-Myc-His detected in the cell lysate (35 ± 5% increase, n = 3; Fig. 1A, lane 3). The observed increase in TXA2R-Myc-His quantity present in the cell lysate after H2O2 treatment suggests that the receptor stabilization may occur rapidly after oxidative stress, similarly to that found in vivo. Further, TXA2R wild type was visualized by immunofluorescence microscopy using rabbit anti-TXA2R C-terminal antibody and anti-rabbit fluorescein isothiocyanate conjugate. Immunofluorescence microscopy showed that in permeabilized cells, TXA2R wild type was distributed throughout the cell, mainly on a reticular network, suggesting an ER localization (Fig. 1B).

To investigate whether oxidative stress could induce some morphology modifications, the cells were observed under electron microscopy before (Fig. 2A) and after oxidative stress (Fig. 2B). No gross modification to the morphology of the cell was observed by thin section electron microscopy, following H2O2 treatment under the conditions used here.

**The Intracellular Localization of TXA2Rβ Is Not a Consequence of the Transient Expression**—To investigate whether the intracellular localization of TXA2Rβ was a consequence of the expression system, we transfected TXA2Rβ into an inducible mammalian expression system. Using the inducible mammalian expression vector (pEGSH vector) containing TXA2Rβ, we transiently transfected pERV3 CHO stable cells in the presence of different concentrations of the inducer, ponasterone A (0–10 μM). Western blot analysis revealed that interaction between the inducer and the ligand-binding domain of the pEGSH vector induced a dose-responsive expression of TXA2Rβ (Fig. 3A, first six lanes). Control (homogenate of transiently transfected COS-7 cells; Fig. 3A, seventh lane) indicated a similar level of expression to that in pERV3 CHO cells in presence of 8 μM of ponasterone A (Fig. 3A, fifth and seventh lanes). To investigate the intracellular localization of TXA2Rβ at low level of expression, transiently transfected cells were maintained in presence of ponasterone A (2 μM) and visualized by immunofluorescence microscopy (Fig. 3B). The nuclei were stained in blue, and TXA2Rβ was stained in red. TXA2Rβ-Myc-His was distributed throughout the cell, mainly on a reticular network, suggesting an ER localization. This localization, which is consistent with the ER, was obtained even with the minimal detectable level of receptor (see the increased signal gain in Fig. 3B, inset). A similar ER localization was also observed in the stably transfected COS-7 cells line (Fig. 4A) or in transiently transfected HCASMc (Fig. 4B) that endog-
These data suggest that the ER localization of TXA2R/H9252 is not a consequence of the artificial expression system.

Oxidative Stress Is Involved in the Intracellular Translocation of TXA2R/H9252—To determine more precisely the subcellular localization of TXA2R/H9252, transiently transfected COS-7 cells were fractionated on nonlinear Nycodenz gradients, using a method previously developed to provide effective separation of ER and Golgi complex proteins (19). As shown in Fig. 5, in the absence of oxidative stress, the main proportion of c-Myc immunoreactivity sedimented to the bottom of the gradient, co-sedimenting with calnexin, a well established ER marker. In contrast, in the presence of H2O2 (10 μM, 40 min), sedimentation of a proportion of TXA2R/H9252 was shifted to a lighter fraction, co-sedimenting with β-COP, a Golgi complex marker (Fig. 5). This observation suggests that upon H2O2 treatment a proportion of the TXA2R/H9252 is translocated to a post-ER compartment.

Oxidative Stress Is Involved in the Stabilization of the TXA2R/H9252—To determine the role of H2O2 in the biogenesis of TXA2R/H9252, we used kinetic analysis. At 24 h post-transfection, cycloheximide was used to abolish protein synthesis; TXA2R/H9252 was localized by confocal fluorescence microscopy. At steady state, confocal microscopy showed that a large proportion of TXA2R/H9252 was localized to the ER (Fig. 6A), and no significant co-localization between TXA2R/H9252 and Golgin-97, a resident Golgi membrane protein, was apparent. In contrast, the addition of H2O2 evoked an intracellular translocation of the TXA2R/H9252 from the ER to the Golgi complex inducing a partial co-localization with Golgin-97 (Fig. 6B, yellow). These results are consistent with the previous observations based on subcellular fractionation suggesting a rapid translocation of the receptor to a post-ER compartment.

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**Fig. 7. Oxidative stress is involved in the stabilization of the TXA2Rβ.** COS-7 cells were transiently transfected with TXA2Rβ-Myc-His, and cell homogenates were analyzed by immunoblotting using anti-Myc antibodies. Kinetic analysis using cycloheximide was performed to investigate the effect of a H2O2 treatment in the absence of BFA (A) or in the presence of BFA (B) on the half-life of TXA2Rβ. The upper panels show representative raw data, with tubulin used as a loading control. The lower panels show quantification of the data normalized at t = 0 to 100%. The data suggest that oxidative stress increases the half-life of the TXA2Rβ pool (A). This observed stabilization is abolished by BFA (B), suggesting that TXA2Rβ stabilization is a consequence of progressing to the Golgi apparatus. The data are representative of three experiments.

**Fig. 8. Effect of BFA treatment on the TXA2Rβ intracellular localization.** COS-7 cells were transiently transfected with TXA2Rβ-Myc-His. The nuclei were stained with DAPI (blue), TXA2Rβ was stained with anti-Myc Cy3 conjugate (red), and the Golgi complex was stained with anti-Golgin-97 (cis-Golgi marker) and an Oregon Green-conjugated secondary antibody (green). Without H2O2 treatment (A), TXA2Rβ-Myc-His is detected in structures distributed throughout the cytoplasm with limited co-localization with the cis-Golgi complex marker (yellow). By contrast, in the presence of H2O2 treatment (B), TXA2Rβ-Myc-His was localized in a very restricted area close to the nucleus showing a co-localization with the cis-Golgi complex marker (yellow). Some TXA2Rβ-Myc-His was in the ER but below the detection limit. These results confirm that oxidative stress induces a translocation of the receptor to the Golgi complex. BFA treatment on oxidatively stressed cells induces a relocalization of the receptor to the ER (C). The disappearance of the Golgi complex staining is due to redistribution of the Golgi complex proteins induced by BFA.
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Fig. 9. Involvement of the UPR in the TXA2Rβ stabilization. COS-7 cells were transiently transfected with TXA2Rβ-Myc-His and cell homogenates analyzed by immunoblotting using anti-Myc antibodies. Kinetic analyses of BiP (A), the effect of DTT on cycloheximide-induced-TXA2Rβ degradation (B), and the effect of tunicamycin on H2O2-induced-TXA2Rβ stabilization (C) were performed. The data show that H2O2 induces an up-regulation of BiP expression with a peak reached after 30 min of H2O2 exposure. DTT induced stabilization of TXA2Rβ, whereas tunicamycin abolished the H2O2-induced TXA2Rβ stabilization. These results indicate that the UPR is most likely involved in the TXA2Rβ stabilization process and that N-glycosylation also plays a crucial role.

was chased for different time periods, before Western blot analysis. Fig. 7A shows that in the absence of H2O2, TXA2Rβ is more rapidly degraded than in the presence of H2O2. After 5 h, TXA2Rβ decreased by 80 ± 5% (n = 3). In contrast, in the presence of H2O2, TXA2Rβ degradation occurred less rapidly, and only 25 ± 5% of TXA2Rβ was degraded after 5 h (n = 3), suggesting that oxidative stress is able to mediate TXA2Rβ stabilization. This is consistent with the observation in Fig. 1A where H2O2 treatment increased the amount of TXA2Rβ detected.

One of the effects of H2O2 on the TXA2Rβ is an increase of the proportion of the TXA2Rβ in the Golgi complex. We attempted to determine whether the Golgi complex localization had a role in TXA2Rβ stabilization. Identical TXA2Rβ kinetic experiments to those described above were performed in the presence of brefeldin A (Fig. 7B). BFA treatment abolished the TXA2Rβ stabilization induced by the H2O2 (93 ± 5% of degradation after 5 h in presence of BFA versus 25 ± 5% in the absence of BFA), without affecting the basal level of TXA2Rβ (Fig. 7B). Immunofluorescence microscopy showed that in quiescent cells, TXA2Rβ was located mainly in the ER compartment (Fig. 8A). Oxidative stress induced a translocation to the Golgi complex area with an ER staining below the detection limit (Fig. 8B). This translocation is partially ablated by the BFA treatment, which causes disassembly of the Golgi complex (Fig. 8C). In immunofluorescence, essentially no receptors were observed on the plasma membrane, which could be explained by a low receptor density on the plasma membrane of permeabilized cells. Taken together, these results suggest that H2O2 stabilizes TXA2Rβ by an intracellular translocation from the ER to the Golgi complex.

Possible Involvement of the UPR in the TXA2Rβ Stabilization—A potential mechanism for rapid turnover of ER-located TXA2Rβ is ER-associated degradation. This pathway is stimulated under numerous stress conditions invoking the UPR. To investigate whether H2O2 exposure could induce the UPR, we evaluated the influence of peroxide on expression of BiP, which is a well-characterized UPR gene in mammalian cells (22). A time course of BiP induction upon exposure of cells to 10 μM is shown in Fig. 9A. H2O2 induces an up-regulation of BiP with a peak (4–5-fold) of induction at 30 min (Fig. 9A, fourth lane). No induction was observed in the absence of oxidative stress (Fig. 9A, seventh lane). Exposure to DTT (2 mM, 6 h; Fig. 9B), which is known to be a strong inducer of UPR (23), prevented the TXA2Rβ degradation induced by 3 h of cycloheximide treatment (65 ± 5% remaining after 3 h in presence of DTT versus 30 ± 5% in the absence of DTT; Fig. 9B, third and fourth lanes). In contrast, cell treatment with tunicamycin (10 μg/ml, 6 h), a further UPR inducer in mammalian cells (23), increased the degradation of TXA2Rβ induced by cycloheximide (75 ± 5% remaining after 2.5 h in absence of tunicamycin versus 20 ± 5% in presence of tunicamycin; Fig. 9C, second and third lanes). In addition, the presence of tunicamycin abolished the stabilization of TXA2Rβ elicited by H2O2 (75 ± 5% remaining after 2.5 h in absence of tunicamycin versus 35 ± 5% in presence of tunicamycin; Fig. 9C, fourth and fifth lanes). Taken together, these results suggest that UPR might be involved in the TXA2Rβ stabilization process and that N-glycosylation also seems to play a crucial role in the control of TXA2Rβ stability.

Effect of the H2O2 Treatment on the Ligand Binding Capacity—A binding assay was performed on an isolated membrane fraction of COS-7 cells using the TXA2R antagonist [3H]SQ29548. Data show that, in the membrane fraction, H2O2 treatment increased [3H]SQ29548-specific binding on the
membrane fraction (B_max = 3.1 ± 0.3 versus 2.3 ± 0.3 pmol/mg protein, p = 0.046, n = 3) without any effect on its affinity (K_d = 7.1 ± 0.6 versus 6.3 ± 0.6 nM, n = 3; Fig. 10).

**DISCUSSION**

Most proteins entering the secretory pathway, including TXA2Rβ, are folded within the ER following translocation via the Sec61 complex. To support efficient folding, the ER maintains a luminal environment enriched in chaperone proteins, glycosylation enzymes, oxidoreductases, and quality control systems (24). Despite this optimized environment, an inevitable consequence of the large flux of proteins through the ER, plus the complexity of folding, is that rather less than 100% of nascent polypeptides attain the native state, resulting in the production of unfolded proteins. The cells respond to an accumulation of unfolded proteins in the ER by increasing transcription of genes encoding several ER resident proteins. Information on folding status is transmitted from the ER lumen to the nucleus by an intracellular signaling pathway involving the UPR, involving N-linked glycosylation of ER proteins (31). All prostanoid receptors have conserved N-glycosylation sites in the N-terminal domain and one of the extracellular loops. Site-directed mutagenesis studies analyzing the function of glycosylation sites revealed that the requirement of N-glycosylation for correct sorting to the plasma membrane is crucial (32). ER quality control is highly dependent upon covalent attachment of the oligosaccharide Glc3Man9GlcNAc2 to specific asparaginyl residues of nascent ER proteins. This requires the synthesis of a lipid-linked oligosaccharide, Glc3Man9GlcNAc2–P-P-dolichol. Tunicamycin is a specific inhibitor of the synthesis of this lipid-linked oligosaccharide and a strong UPR inducer (23). Our results indicate that despite the fact that tunicamycin induces the UPR, it was not able to prevent TXA2Rβ degradation and abolished the TXA2Rβ stabilization induced by H2O2. This result supports the model that receptor glycosylation is essential for correct folding and translocation to the plasma membrane as previously shown on cell lines naturally expressing a prostaglandin E1 receptor (33). Because TXA2Rβ synthesized in the presence of tunicamycin will not be N-glycosylated, it is likely that correct, efficient folding of the receptor requires interaction with calnexin. Prevention of calnexin interaction will accelerate the rate at which TXA2Rβ folding intermediates are rejected by the ER quality control system, abating the stabilization normally observed with peroxide treatment.

Our results indicate that oxidative stress induces maturation and stabilization of an unstable intracellular TXA2Rβ pool, most likely by translocation to the Golgi complex. Importantly, these observations also suggest that TXA2Rβ levels are modulated by ER-associated proteolysis and controlled by the efficiency of transport to post-ER compartments. This stabilization of TXA2Rβ by translocation from a degradative compartment may account for the rapid augmentation of receptor density observed in vivo and for the potentiation of the 8-isoprostaglandin F2α-induced vasoconstriction during oxidant injury.

**Acknowledgments**—We thank Drs. J. A. Ware and A. W. Ashton for providing cDNA encoding TXA2Rβ, David Goulding for electron microscopy technical assistance, Prof. Keith Gull for anti-tubulin antibody, and Dr. Bassem Ali for insightful discussions about many aspects of this work.

**REFERENCES**

1. FitzGerald, G. A., Healy, C., and Daugherty, J. (1987) *Fed. Proc.*, **46**, 154–158
2. Narumiya, S., and Sugimori, H. (1999) *Physiol. Rev.*, **79**, 1193–1226
3. Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakashishi, S., and Narumiya, S. (1991) *Nature* **349**, 617–620
4. Raychowdhury, M. K., Yukawa, M., Collins, L. J., McGrail, S. H., Kent, K. C., and Ware, J. A. (1994) *J. Biol. Chem.* **269**, 19256–19261
5. Smrcka, A. V., and Sternweis, P. C. (1993) *J. Biol. Chem.* **268**, 9676–9674
6. Morrow, J. D., Harris, T. M., and Roberts, L. J. II (1990) *Anal. Biochem.* **184**, 87–90
7. Mehrabi, M. R., Ekmekcioglu, C., Tatscher, F., Ogugho, A., Ulrich, R., Morgan, A., Tamaddon, F., Grimm, M., Glogar, H. D., and Sinzinger, H. (1999) *Cardiovasc. Res.* **35**, 490–499
8. Kromer, B. M., and Tippins, J. R. (1996) *Br. J. Pharmacol.* **119**, 1276–1280
9. Audoly, L. P., Rocca, B., Faure, J. E., Koller, B. H., Thomas, D., Loe, A. L., Coffman, T. M., and FitzGerald, G. A. (2000) *Circulation* **101**, 2833–2840
10. John, G. W., and Valentine, J. P. (1997) *Br. J. Pharmacol.* **122**, 899–905
11. Kromer, B. M., and Tippins, J. R. (1999) *Br. J. Pharmacol.* **126**, 1171–1174
12. Kamekura, I., Okumura, K., Matsumi, H., Murase, K., Mokuno, S., Toki, Y., Nakashima, Y., and Itô, T. (1999) *J. Cardiovasc. Pharmacol.* **34**, 836–842
13. Podesta, P. A., Colella, A., Cecioni, I., Costoli, A., Biagini, D., Migliorini, A., and Neri Serneri, G. G. (1995) *Am. Heart J.* **129**, 873–879
14. Katugampola, S. D., Kuc, R. E., Maguire, J. J., and Davenport, A. P. (2002) Clin. Sci. 103, 171S-175S.
15. Katugampola, S. D., and Davenport, A. P. (2001) Br. J. Pharmacol. 134, 1385–1392
16. Sun, D., Nguyen, N., DeGrado, T. R., Schwaiger, M., and Brosius, F. C., III (1994) Circulation 89, 793–798
17. Halaban, R., Cheng, E., Svedine, S., Aron, R., and Hebert, D. N. (2001) J. Biol. Chem. 276, 11933–11938
18. Gething, M. J., and Sambrook, J. (1992) Nature 355, 33–45
19. Rickwood, D., Ford, T., and Graham, J. (1982) Anal. Biochem. 123, 23–31
20. Becker, K. P., Ullian, M., and Halushka, P. V. (1998) Biochim. Biophys. Acta 27, 109–114
21. Morinelli, T. A., Zhang, L. M., Newman, W. H., and Meier, K. E. (1994) J. Biol. Chem. 269, 5693–5698
22. Sato, N., Urano, F., Yoon, L. J., Kim S. H., Li, M., Donoviel, D., Bernstein, A., Lee, A. S., Ron, D., Veselits, M. L., Sisodia, S. S., and Thinakaran, G. (2000) Nat. Cell Biol. 2, 863–870
23. Benedetti, C., Fabbri, M., Sitia, R., and Cabisbo, A. (2000) Biochem. Biophys. Res. Commun. 278, 530–536
24. Ellgaard, L., Molinari, M., and Helenius, A. (1999) Science 286, 1882–1888
25. Chapman, R., Sidrauski, C., and Walter P. (1998) Annu. Rev. Cell Dev. Biol. 14, 459–485
26. Kapito, R. R. (1999) Physiol. Rev. 79, 8167–173
27. Yoshimura, A., D'Andrea, A. D., and Lodish, H. F. (1990) Proc. Natl. Acad. Sci. U. S. A. 11, 4139–4143
28. Donaldson, J. G., Lippincott-Schwartz, J., Bloom, G. S., Kreis, T. E., and Klausner, R. D. (1990) J. Cell Biol. 111, 2295–2306
29. Chang, N., and Tai, H. H. (1998) Arch. Biochem. Biophys. 352, 207–213
30. Sousa, M., and Parodi, A. J. (1995) EMBO J. 14, 4196–4203
31. Shang, J., Koerner, C., Freeze, H. H., and Lehrman, M. A. (2002) Glycobiology 12, 307–317
32. Boer, U., Neuschaefer-Rube, F., Moller, U., and Puschel, G. P. (2000) Biochem. J. 350, 839–847
33. Yatsunami, K., Fujisawa, J., Hashimoto, H., Kimura, K., Takahashi S., and Ichikawa, A. (1990) Biochim. Biophys. Acta 1031, 94–99

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