Establishment of an *in vivo* model facilitates B2 receptor protein maturation and heterodimerization

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In individuals with diverse cardiovascular risk factors, signalling stimulated by the AT_1 receptor for the vasopressor angiotensin II is sensitized by heterodimerization with the receptor for the vasodepressor bradykinin, B_2. Signal sensitization and receptor heterodimerization rely on efficient maturation of the B_2 receptor protein. To assess functional features of that important cardiovascular receptor system, we established an *in vivo* model by using immunodeficient NOD.Scid mice for the expansion of transfected cells under physiological conditions. Compared to cultivated cells, the *in vivo* model strongly facilitated B_2 receptor maturation and heterodimerization. To elucidate the mechanisms underlying the enhancement of B_2 receptor protein maturation under *in vivo* conditions, we performed microarray gene expression profiling. Microarray analysis revealed a more than 1.7-fold up-regulation of the chaperone calreticulin upon *in vivo* cell expansion whereas other important members of the general chaperone system were only marginally altered. Down regulation of calreticulin expression by RNA interference confirmed the importance of calreticulin for efficient B_2 receptor maturation under *in vivo* conditions. Receptor proteins synthesized in the Nod.Scid cell expansion model were functionally active and sensitive to drug treatment as exemplified by treatment with the AT_1-specific antagonist losartan. Thus, we established a model system that can be used to analyze functional features of proteins *in vivo* by expanding transfected cells in immunodeficient NOD.Scid mice.

Introduction

The major receptor for the vasoactive peptide hormone angiotensin II, AT_1, exerts an indispensable physiological role in regulating vascular tone, ion and water homeostasis. Apart from the important physiological role, the pathogenesis of many cardiovascular disorders is characterized by a dysregulation of the vital angiotensin II AT_1 receptor system leading to an exaggerated angiotensin II response.[1] Cellular mechanisms accounting for the hyperactivity of the angiotensin II system are therefore a major focus of research aimed to improve strategies for the treatment of cardiovascular disease.

Interaction of the bradykinin B_2 receptor with the angiotensin II AT_1 receptor occurs *in vivo* and may contribute to the hyperactivity of AT_1-stimulated signal pathways under pathological conditions of cardiovascular disorders.[2–5] Signal enhancement of the AT_1 receptor by the B_2 receptor relies on the formation of AT_1/B_2 receptor heterodimers, which requires disulfide-bond formation.[3,4] The covalent association of AT_1 and B_2 receptors seems to account for the enhanced G-protein activation of AT_1/B_2 heterodimers relative to dissociable receptors because covalently stabilized receptors

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Insight, innovation, integration

The AT_1 receptor for the vasopressor angiotensin II is involved in the pathophysiology of hypertension and cardio-vascular disorders. Under various *in vivo* conditions, the AT_1 response is sensitized by heterodimerization with the B_2 receptor. To analyze functional features of that important cardiovascular receptor system, the study established a novel model that applies immunodeficient Nod.Scid mice for expansion of cultured cells *in vivo*. Compared to conventional cell culture conditions, receptor protein maturation and heterodimerization were strongly facilitated by the novel model. In search for specific features of the system, microarray gene expression profiling revealed fine-tuned alterations of the general chaperone system that accounted for enhanced protein maturation and function in the novel cell expansion model.

constitute a cellular platform that is kinetically favoured to interact with and activate heterotrimeric G-proteins.[6,7] In agreement with this concept, signal enhancement of AT_1 by the B_2 receptor does not require the binding of bradykinin to B_2 because a mutated B_2 receptor with a ~ 700-fold reduced affinity for bradykinin is still capable of enhancing AT_1 receptor-stimulated G-protein activation and signalling.[3] Likewise, a B_2 specific antagonist did not interfere with the angiotensin II-mediated activation of AT_1/B_2 receptor heterodimers.[4] In contrast, a B_2 receptor mutant deficient in G-protein activation did not enhance AT_1 receptor-stimulated signalling.[3] These findings strongly suggest that the intracellular receptor interface of B_2 contacting the G-protein is important for the signal enhancement of AT_1.

In addition to signal enhancement, AT_1/B_2 heterodimerization also alters the pathway of receptor internalization: The dynamin-independent internalization of individual AT_1 and B_2 receptors is switched to a dynamin-dependent internalization pathway upon AT_1/B_2 heterodimerization.[3] Together these data point to conformational changes of the intracellular receptor interface upon heterodimerization, which may be responsible for the specific features of AT_1/B_2 heterodimers distinguishing receptor (hetero-)dimers from dissociable receptors.[6,7]

The specific characteristics of AT_1/B_2 receptor heterodimers were mainly deduced from receptors expressed under native conditions.[2–5] In contrast to native conditions, receptors synthesized from transfected genes often contain large quantities of immature protein prone to aggregation because the endogenous chaperones assisting protein folding may be limiting.[8,9] To further analyze the functional features of AT_1/B_2 receptor heterodimers and their potential (patho-) physiological role, we established a novel model that recapitulates protein folding under in vivo conditions by expanding cultured cells in immunodeficient NOD.Scid mice.

Materials and methods

Cultivation of cells and cell transfection

HEK293 cells were routinely grown in DMEM (450 mg/dl glucose) supplemented with 10% (v/v) FCS, and kept in a humidified 7.5% CO_2/92.5% air atmosphere at 37 °C unless otherwise indicated. Cells were transfected with Lipofectamine Plus (Invitrogen). Plasmids encoding the human B_2 and AT_1 receptor under control of the CMV promoter (pcDNA3, Invitrogen) were used to generate clonal cell lines with stable expression. To down regulate calreticulin expression by RNA interference (RNAi), replated NOD.Scid-expanded HEK293 cells were transfected with stealth RNAi[10] targeting the coding sequence of the human calreticulin cDNA (nucleotides 59–83, RNAi-Calreticulin 1; nucleotides 320–344, RNAi-Calreticulin2).[10] Down regulation of calreticulin was assessed by immunoblotting 40 h after transfection. For RNA interference studies under in vivo conditions, cell clones with RNA polymerase II promoter-driven expression of a control micro-RNA or a micro-RNA targeting calreticulin (nucleotides 324–344) were expanded in NOD.Scid mice.[11]

Membrane preparation

Membranes of in vitro cultivated or in vivo expanded HEK293 cells were prepared by sucrose density gradient centrifugation as described.[3] Briefly, the crude membrane pellet of in vitro cultivated or in vivo expanded cells was prepared in 20 mM Hepes, pH 7.4 containing 1 mM phenylmethylsulfonyl fluoride, 2 µM enalaprilate, 2 µM leupeptin, 1 mM bacitrin, 1 µM E64 and 1 mM phosphoramidon, layered onto a gradient composed of 10–45% (w/v) sucrose and centrifuged ($100\,000 \times g$, 4 °C, 4 h). The membrane fraction was collected, diluted to a protein concentration of 1 mg ml^{-1} and stored at −80 °C for further use. Receptor-enriched membranes contained usually ~ 0.5–1 pmol B_2 receptor/mg protein unless otherwise indicated.

Antibodies used for immunoblotting and immunofluorescence

The following antibodies were used for immunoblotting, receptor immunoaffinity enrichment and immunofluorescence: affinity-purified rabbit/rat anti-B_2 receptor antibodies (raised against an antigen corresponding to positions 28–60 of the human B_2 receptor sequence); affinity-purified rabbit/rat anti-B_2 receptor antibodies (raised against an antigen corresponding to positions 356–391 of the human B_2 receptor sequence); affinity-purified rabbit/rat anti-AT_1 receptor antibodies (raised against an antigen corresponding to positions 306–359 of the human AT_1 receptor sequence); affinity-purified rabbit anti-AT_2 receptor antibodies (raised against an antigen corresponding to positions 320–349 of the human AT_2 receptor sequence). Specificity and cross-reactivity of the resulting antisera with the respective protein was routinely monitored by immunoblotting and immunofluorescence. All antibodies were characterized in previous studies.[3–6,11,12]

Protein detection in immunoblot and co-enrichment of receptors

Immunoblotting was performed with membranes prepared by sucrose density gradient centrifugation as described above followed by partial enrichment.[3] Proteins were dissolved in SDS-sample buffer containing 2% SDS, 5% β-mercaptoethanol and 6 M urea for 30 min at room temperature. Protein samples were separated by SDS-PAGE under reducing conditions and supplemented with urea followed by transfer to PVDF membranes. Affinity-purified antibodies or F(ab)$_2$ fragments of the respective antibodies pre-absorbed to human proteins were used for detection of B_2 or AT_1 receptors. Bound antibody was visualized by pre-absorbed F(ab)$_2$ fragments of enzyme-coupled secondary antibodies or by enzyme-coupled Protein A followed by enhanced chemiluminescence detection (ECL plus).

For co-enrichment of AT_1 and B_2 receptors, membranes of explanted HEK cells were solubilized with RIPA buffer (including protease inhibitor cocktail), and subjected to immunoaffinity chromatography by anti-AT_1 receptor antibodies using 0.1 ml immunoaffinity matrix (Affigel 10; 15 mg affinity-purified antibodies per ml gel). After extensive washing, proteins were eluted with 0.2 M glycine, pH 2.5, neutralized, desalted, delipidated and precipitated.[3] Eluted proteins were dissolved and separated by urea-containing SDS-PAGE under reducing conditions. Enriched AT_1 and

co-enriched B_2 receptors were identified in immunoblot with the respective anti-receptor antibodies.

Immunofluorescence

Detection of receptors on NOD.Scid-expanded HEK293 cells was performed with dispersed cells expanded in NOD.Scid mice or cryosections (10 μm) of the isolated and frozen HEK cell pellet obtained from 3 months-old NOD.Scid mice three weeks after subcutaneous injection of 6–8×10^6 HEK293 cells/200 μl. For co-localization of AT_1 and B_2, affinity-purified rat anti-AT_1 receptor antibodies and rabbit anti-B_2 receptor antibodies were applied (dilution 1 : 4000), followed by secondary antibodies labeled with Alexa Fluor 488 and Alexa Fluor 546, respectively (Molecular Probes; dilution 1 : 5000). Sections and cells were imaged with a Leica DMI6000 and a confocal laser microscope (Leica TCS SPE).

Microarray gene expression profiling

For microarray gene expression analysis, replated NOD.Scid-expanded HEK293 cells, and in vitro cultivated HEK293 cells (cultivated in DMEM supplemented with 10% FCS, and 450 mg/dl or 100 mg/dl glucose as indicated) were used. Total RNA was isolated with the RNeasy Mini kit (Qiagen). Procedures for cDNA synthesis, labeling and hybridization were carried out according to the protocol of the manufacturer (Affymetrix GeneChip Expression Analysis Technical Manual; Rev. 5). For hybridization, 15 μg of fragmented cRNA were incubated with the chip (Affymetrix GeneChip Human genome U133 Plus 2.0 Array) in 200 μl of hybridization solution in a Hybridization Oven 640 (Affymetrix) at 45 °C for 16 h. GeneChips were then washed and stained using the Affymetrix Fluidics Station 450. Microarrays were scanned with the Affymetrix GeneChip Scanner 7G, and the signals were processed using GCOS (v. 1.4, Affymetrix). Gene expression data are available at NCBI GEO database accession no GSE15575 and GSE18739.

In vivo expansion of HEK293 cells in NOD.Scid mice

NOD.Scid mice (age 3 months) were injected subcutaneously with 6–8×10^6 cells/200 μl PBS. For expansion in NOD.Scid mice, HEK293 cell clones were used stably expressing the B_2 receptor (HEK-B2) or co-expressing AT_1 and B_2 receptors (HEK-AT1/B2). Three weeks after the injection, mice were anesthetized, perfused intracardially with physiological phosphate buffer, pH 7.2, and the expanded HEK293 cell pellet was isolated and processed for further use. As indicated, NOD.Scid mice were treated with or without the AT_1-specific antagonist, losartan, for three weeks (30 mg kg^{-1} supplied in drinking water).

Quantification of cell-surface B_2 receptors was performed on dispersed, in vivo expanded HEK cells with 50 nM of [^{125}I]-labeled F(ab)$_2$ fragments of affinity-purified B_2 receptor-specific antibodies ($\sim$1 μCi) similarly as described.[11]

Animal experiments

All animal experiments were reviewed and approved by the committees on animal research at the University of Hamburg

and Zurich, and were conducted in accordance with the NIH guidelines.

Statistics

Unless otherwise stated, data are expressed as mean ± S.E. To determine significance between two groups, we made comparisons using the unpaired, two-tailed Student's t-test. P values <0.05 were considered significant.

Results

Maturation of the bradykinin B_2 receptor protein in cultivated HEK293 cells depends on cell and culture conditions

AT_1/B_2 receptor heterodimerization relies on efficient maturation of the B_2 receptor protein.[10] Over-expressing cells often produce large amounts of immature protein because the endogenous pool of chaperones may be limiting.[8,9] In agreement with those observations, HEK293 cells over-expressing the B_2 receptor synthesized significant amounts of an immature B_2 receptor protein of 53 ± 4 kDa in addition to the mature protein of 67 ± 5 kDa when cultivated in a standard cell culture medium containing a glucose concentration of 450 mg/dl (Fig. 1(a), lane 1; and ref. 10).

Glucose is known to down regulate the expression of several chaperones.[13] To enhance the maturation of the B_2 protein, we applied a medium with a "physiological" (low) glucose concentration of 100 mg/dl. Compared to high glucose, the low glucose medium led to the enhanced synthesis of the mature B_2 receptor protein of $\sim$67 kDa (Fig. 1(a), lane 2; and ref. 10). These observations show that B_2 receptor protein maturation of in vitro cultivated cells is highly dependent on cell and culture conditions.

Identification of glucose-sensitive members of the general chaperone system by microarray gene expression profiling

To determine the impact of cell culture conditions on the general chaperone system of HEK293 cells, we performed microarray gene expression profiling of HEK293 cells cultivated in medium with high and low glucose, respectively. Microarray gene expression profiling revealed a strong up-regulation of the general chaperone system of HEK293 cells cultivated in low glucose medium relative to high glucose (Fig. 1(b) and ref. 10). The 14 glucose-sensitive members of the general chaperone system of HEK293 cells are depicted in Fig. 1(b).

Immunoblot analysis confirmed the increased expression of important members of the general chaperone system under low glucose as exemplified for GRP78 and calreticulin (Fig. 1(c)). As a control, β-actin expression levels were not significantly different (Fig. 1(b and c)). Thus, maturation of a prototypic membrane protein can be severely affected by the chosen glucose concentration of the culture medium because several members of the general chaperone system are highly glucose-sensitive.

In vivo expansion of HEK293 cells in NOD.Scid mice restores an imbalanced chaperone system

As demonstrated above with a prototypical membrane receptor, protein maturation can be extremely variable
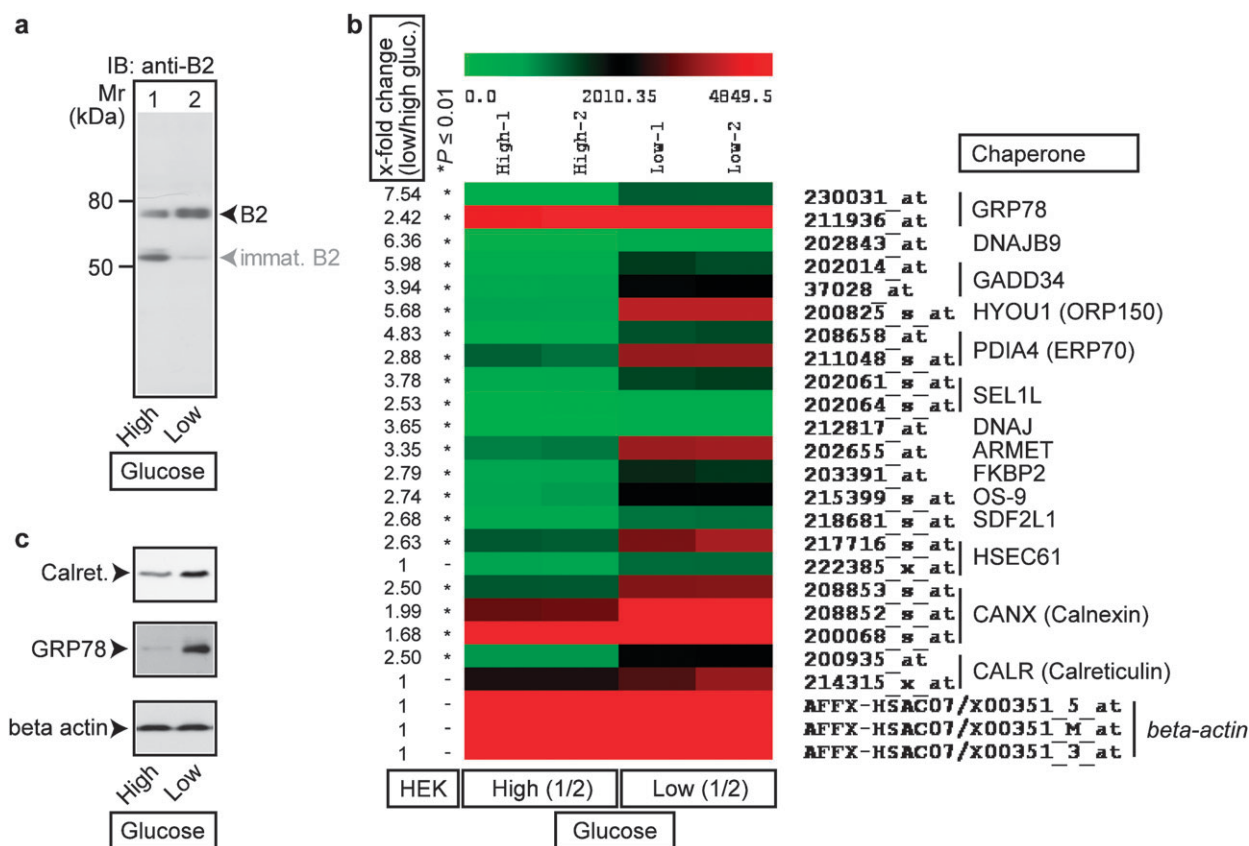
Fig. 1 Microarray gene expression profiling identifies glucose-sensitive chaperones of HEK293 cells. (a) Immunoblot detection of the B_2 receptor (IB: anti-B2) on enriched membranes of HEK293 cells (expression of $\sim$200 fmol B_2/mg protein) cultivated in a medium with high (450 mg/dl) or low (100 mg/dl) glucose revealed the mature (B2) and immature B_2 receptor protein (immat. B2). (b) Microarray gene expression profiling of HEK293 cells. Differentially expressed probe sets of low glucose-cultivated HEK293 cells relative to high glucose-cultivated HEK293 cells (x-fold change; low/high gluc.; *P value ≤ 0.01), ER localization and involvement in the general chaperone system are listed. If available, alternative probe sets of the differentially expressed chaperones are also shown. Normalized signal intensity values are presented as heat map. Probe sets of beta actin demonstrate integrity of biotin-labeled cRNAs used for gene chip hybridization ($3'/5'$ ratio: 1.17–1.36). (c) Immunoblot analysis controlling the microarray data.

depending on the selected culture conditions. To analyze protein maturation in a more standardized environment, we established an *in vivo* model. To this end we used immuno-deficient NOD.Scid mice for *in vivo* expansion of HEK293 cells. Cells were injected subcutaneously, and after three weeks, the expanded HEK293 cell pellet was isolated from NOD.Scid mice (Fig. 2(a)).

In contrast to conventional cell cultivation, *in vivo* expanded cells are exposed to a tightly controlled physiological environment. To validate the novel model, we analyzed the general chaperone system by microarray gene expression profiling. For our analysis, we selected those chaperones that were highly regulated under *in vitro* cell culture conditions (Fig. 2(b) *versus* Fig. 1(b)). Interestingly, the *in vivo* model showed only subtle alterations of the general chaperone system relative to *in vitro* cultured HEK293 cells (Fig. 2(b)). A significantly different expression was only detected for probe sets of four different chaperones, *i.e.* glucose-regulated protein (GRP78), protein disulfide isomerase (PDIA4), HSEC61 and calreticulin (Fig. 2(b)). With a more than 1.7-fold increased expression, calreticulin showed the strongest difference between *in vivo* and *in vitro* conditions. Thus, the

in vivo expansion of cells in NOD.Scid mice apparently restored an imbalanced chaperone system without inducing a massive up-regulation of the entire protein folding machinery.

Expansion of HEK293 cells in NOD.Scid mice facilitates B_2 receptor protein maturation

The selective enhancement of calreticulin expression in the established model system was intriguing because calreticulin is known to be required for B_2 receptor maturation, hetero-dimerization and function.[10,14] We therefore investigated whether B_2 receptor protein maturation was enhanced upon *in vivo* cell expansion. HEK293 cell clones stably expressing the B_2 receptor were implanted subcutaneously into NOD.Scid mice as detailed above. After three weeks, the expanded cell pellets were isolated. Immunofluorescence confirmed that the isolated cells consisted of expanded HEK293 cells because the B_2 receptor was only detected on expanded HEK-B2 cells stably expressing the B_2 receptor whereas B_2 receptors were not detected on expanded control HEK-P3 cells lacking B_2 receptor expression (Fig. 3(a)).
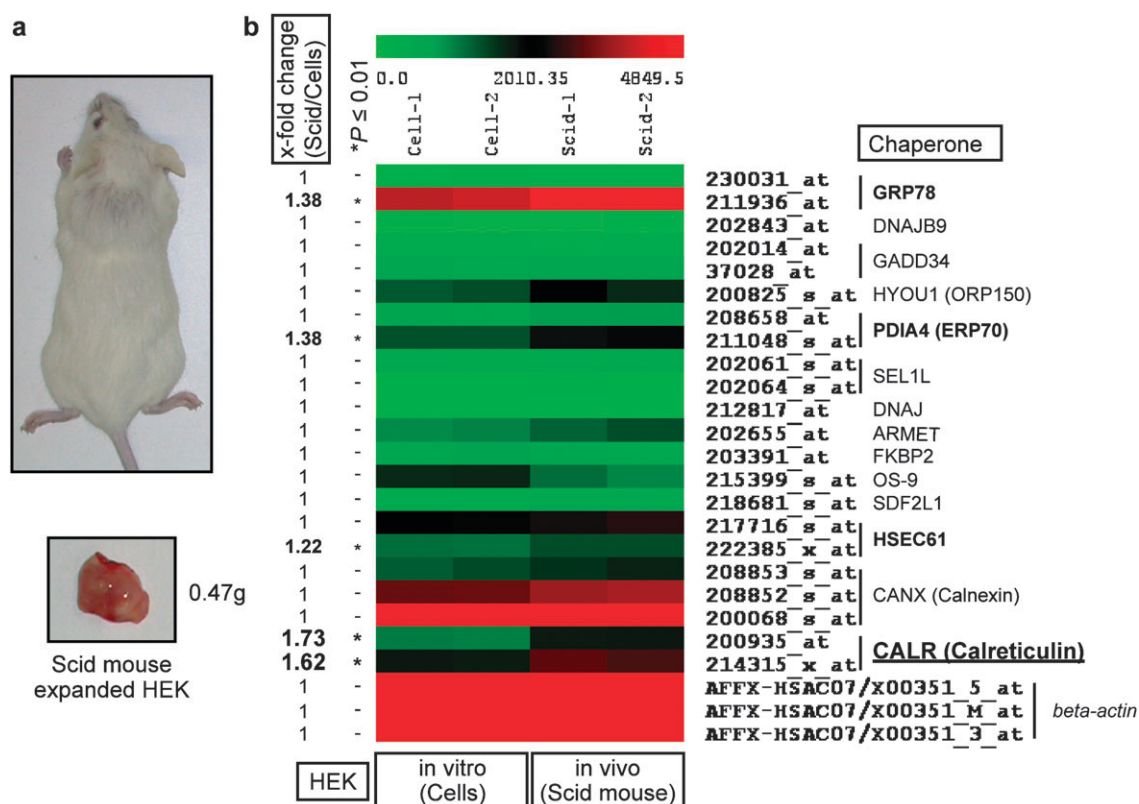
Fig. 2 *In vivo* **expansion of HEK293 cells in NOD.Scid mice restores an imbalanced chaperone system.** (a) HEK293 cells were subcutaneously injected into immune-deficient NOD.Scid mice. After *in vivo* expansion for three weeks, mice were anesthetized (upper panel), and the expanded cell pellet was isolated (lower panel; Scid mouse-expanded HEK). (b) Microarray gene expression profiling of NOD.Scid-expanded (*in vivo*; Scid mouse), and *in vitro* cultivated clonal HEK293 cells (*in vitro*, Cells; 450 mg/dl glucose). The heat map shows normalized signal intensity values of the glucose-sensitive chaperones identified in Fig. 1(b). The analysis identified differentially expressed probe sets of four different chaperones (*P value ≤ 0.01). Probe sets of beta actin demonstrate integrity of biotin-labeled cRNAs used for gene chip hybridization ($3'/5'$ ratio: 1.23–1.28). The microarray data represent two biological replicates for each condition (GSE18739). Data shown are representative of three independent experiments performed with 2–3 mice each.

Next, we determined the B_2 receptor in immunoblot. In agreement with the previous data (*cf*. Fig. 1(a)), the B_2 receptor of conventionally cultivated HEK293 cells kept in a high glucose medium appeared predominantly as an immature form of 53 ± 4 kDa. In contrast, upon *in vivo* expansion in NOD.Scid mice, the B_2 receptor of HEK293 cells was synthesized as a mature form of 67 ± 5 kDa (Fig. 3(b)). Immunoblot detection of calreticulin confirmed the microarray data showing that the *in vivo* expansion of cells in NOD.Scid mice led to significantly increased calreticulin protein levels relative to *in vitro* cultivated HEK293 cells (Fig. 3(b)). For comparison, changes in GRP78 expression were only minor (Fig. 3(b)). As a control, maturation of the B_2-related angiotensin II AT_2 receptor protein was not different before and after *in vivo* expansion (Fig. 3(c)). Thus, the *in vivo* environment of NOD.Scid mice facilitated maturation of the B_2 receptor protein.

Involvement of calreticulin in B_2 receptor maturation of *in vivo* expanded cells

To analyze whether calreticulin was indeed involved in B_2 receptor maturation, we down-regulated calreticulin expression of *in vivo* expanded and replated HEK cells by transfection of stealth RNAi. Down regulation of calreticulin by RNA interference was confirmed by immunoblot with calreticulin-specific antibodies (Fig. 4(a), left panel). As a control, transfection with an unrelated control RNAi duplex did not affect calreticulin protein levels (Fig. 4(a), left panel). Concomitant to the down-regulation of calreticulin, protein levels of the immature B_2 receptor form of 53 ± 4 kDa were strongly increased (Fig. 4(a), right panel).

Similar results were obtained by RNA interference studies under *in vivo* conditions by expanding two different cell clones with RNA polymerase II promoter-driven expression of a micro-RNA targeting calreticulin (Fig. 4(b)). Upon three weeks of expansion in NOD.Scid mice, micro-RNA expressing HEK cell clones targeting calreticulin by RNA interference showed a significant down regulation of calreticulin expression relative to cell clones expressing a control micro-RNA as assessed by immunoblotting (Fig. 4(b), left panels). In agreement with the involvement of calreticulin in B_2 receptor maturation under *in vivo* conditions, protein levels of the immature B_2 receptor form of 53 ± 4 kDa were significantly increased upon down regulation of calreticulin (Fig. 4(b), right panel). Together these experiments provide evidence that restoration of calreticulin expression contributed to the enhanced B_2 receptor protein maturation of the novel *in vivo* model.
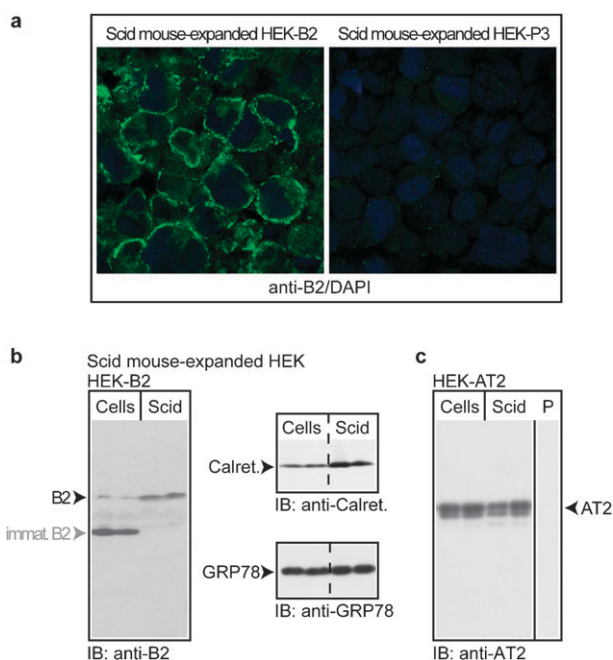
Fig. 3 Expansion of HEK293 cells in NOD.Scid mice facilitates B_2 receptor protein maturation. (a) Immunofluorescence detection of the B_2 receptor with anti-B_2 receptor antibodies (anti-B2; cell nuclei were stained with DAPI) on a cryosection of a NOD.Scid-expanded HEK-B2 cell pellet (left panel; Scid mouse-expanded HEK-B2). As a control, the anti-B_2 receptor antibodies did not interact with expanded HEK-P3 cells lacking B_2 receptor expression (right panel; Scid mouse-expanded HEK-P3), (original magnification, 630×). (b) *In vivo* expansion of two different B_2 receptor expressing HEK-B_2 cell clones for three weeks in NOD.Scid mice induced the synthesis of the mature B_2 receptor of ∼67 kDa (lanes 3,4; Scid) whereas the immature ∼53 kDa B_2 receptor (immat. B2) was the predominant form before *in vivo* expansion and cultivation in high glucose medium (lanes 1,2; Cells). The B_2 receptor was detected on enriched membranes by immunoblot with F(ab)$_2$ fragments of affinity-purified B_2-specific antibodies pre-absorbed to human proteins (IB: anti-B2). Right panels: Calreticulin protein expression as determined in immunoblot with anti-calreticulin antibodies (IB: anti-Calret.) was significantly increased upon *in vivo* expansion of HEK-B2 cells in NOD.Scid mice (upper panel; "Scid" *versus* "Cells"). Immunoblot detection of GRP78 did not reveal a major difference between *in vivo* expanded relative to conventionally cultivated cells (lower panel). (c) As a control, protein levels and maturation of the related AT_2 receptor were not different before and after *in vivo* expansion of two different HEK-AT2 cell clones in NOD.Scid mice as determined in immunoblot with F(ab)$_2$ fragments of affinity-purified anti-AT_2 antibodies pre-absorbed to human proteins (IB: anti-AT2). In lane "P", anti-AT_2 antibodies pre-absorbed to the immunizing antigen were used as a control to confirm antibody specificity.

In vivo expansion of HEK293 cells in NOD.Scid mice enhances AT_1/B_2 receptor heterodimerization

Efficient maturation of the B_2 receptor protein is required for heterodimerization with the AT_1 receptor.[10] To analyze whether the *in vivo* model also supported heterodimerization of the B_2 receptor with the AT_1 receptor, we expanded HEK-AT1/B2 cells co-expressing AT_1 and B_2 receptors in NOD.Scid mice. The expression of the AT_1 and B_2 receptor on *in vivo* expanded HEK cells was determined by immunofluorescence applying antibodies specifically cross-reacting with the AT_1 and B_2 receptor, respectively.[3–5] Immunofluorescence analysis demonstrated a high degree of co-localization of AT_1 and B_2 receptors on the cell membrane of *in vivo* expanded HEK-AT1/B2 cells (Fig. 5(a)).

To determine whether the co-localized receptors formed a direct protein interaction, we enriched the AT_1 receptor with AT_1-specific antibodies, and detected the co-enriched B_2 receptor with B_2-specific antibodies in immunoblot. As a control, efficient enrichment of AT_1 was confirmed by immunoblotting with AT_1-specific antibodies. Enrichment of AT_1 receptors with AT_1-specific antibodies and detection of co-enriched B_2 receptors in immunoblot revealed the direct interaction of AT_1 with B_2 receptors on NOD.Scid-expanded HEK-AT1/B2 cells (Fig. 5(b)). Thus, the mature B_2 receptor protein of *in vivo* expanded cells undergoes a direct interaction with the AT_1 receptor, which is a characteristic feature of receptor hetero(di)merization.[2–5,10]

Assessment of AT_1/B_2 receptor function *in vivo* by drug treatment with the AT_1-specific antagonist losartan

Do the AT_1/B_2 receptor complexes of *in vivo* expanded HEK-AT1/B2 cells display functional features of AT_1/B_2 receptor heterodimers? The functional coupling of the *in vivo* synthesized heterodimers was analyzed by their sensitivity to stimulation with angiotensin II. Stimulation of AT_1/B_2 receptor heterodimers by angiotensin II induces receptor co-internalization and down regulation.[3,15] To assess the stimulation of AT_1/B_2 receptor heterodimers *in vivo*, we determined the effect of circulating blood angiotensin II on B_2 receptors associated with AT_1 in AT_1/B_2 heterodimers relative to individual B_2 receptors. To this end, NOD.Scid mice were transplanted with HEK-AT1/B2 or HEK-B2 cells. After cell injection, mice were treated for three weeks with a standard dose of the AT_1-specific antagonist, losartan (30 mg kg^{-1}, supplied in drinking water), which inhibits AT_1 receptor-stimulated responses in mice.[16] The number of cellular B_2 receptors after three weeks of *in vivo* expansion was quantified with [^{125}I]-labeled F(ab)$_2$ fragments of affinity-purified anti-B_2 receptor antibodies. Losartan treatment of NOD.Scid mice induced a significant increase in the number of B_2 receptors on expanded HEK-AT1/B2 cells relative to untreated mice (Fig. 6, columns 1, 2). For comparison, losartan treatment did not affect B_2 receptor levels of NOD.Scid-expanded HEK-B2 cells expressing only B_2 receptors (Fig. 6, columns 3, 4). As a control, *in vivo* expanded HEK-P3 cells without B_2 receptor expression did not show a significant interaction with [^{125}I]-labeled anti-B_2 receptor antibodies (Fig. 6, columns 5, 6). Together these experiments are compatible with the notion that B_2 receptors, which are part of functional AT_1/B_2 receptor heterodimers are internalized by circulating angiotensin II activating AT_1. *Vice versa*, inhibition of AT_1 by losartan could prevent AT_1/B_2 co-internalization as revealed by the increased B_2 receptor number of HEK-AT1/B2 cells isolated from losartan-treated mice relative to untreated mice.

Altogether, the *in vivo* expansion of HEK cells in NOD.Scid mice restored an imbalance of the general chaperone system induced by conventional *in vitro* cell culture. As a consequence,
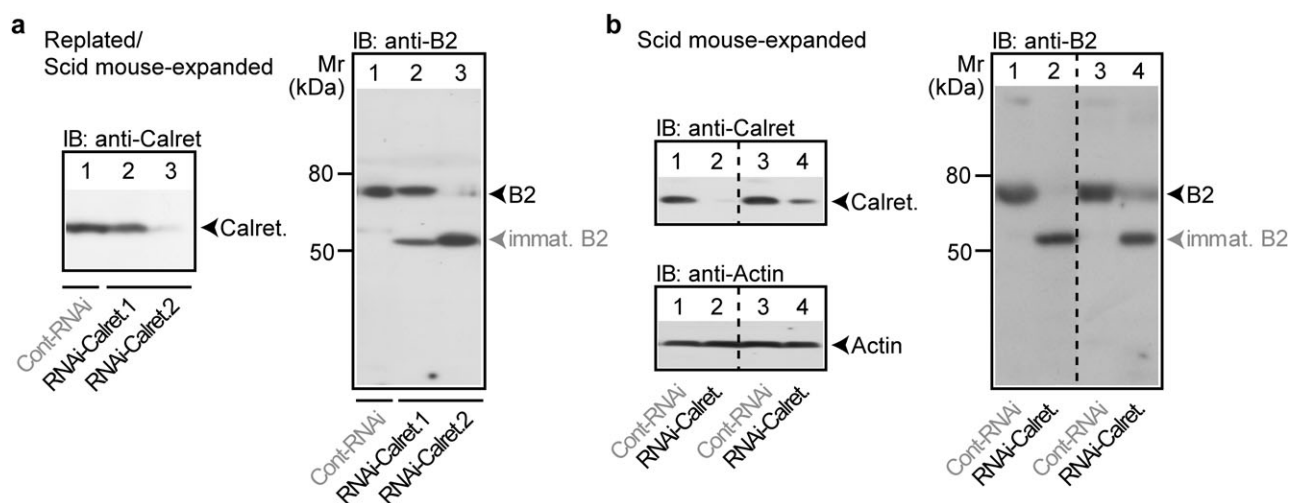
Fig. 4 Involvement of calreticulin in B₂ receptor maturation of *in vivo* expanded cells. (a) Left panel: Immunoblot detection of calreticulin (IB: anti-Calret.) in replated, NOD.Scid-expanded HEK-B2 cells transfected with a control RNAi duplex (Cont-RNAi; lane 1) or RNAi duplexes targeting the coding sequence of calreticulin (RNAi-Calret.1/2; lanes 2,3) confirms the down regulation of calreticulin by RNA interference. Right panel: Immunoblot detection of the B₂ receptor with F(ab)₂ fragments of affinity-purified B₂-specific antibodies pre-absorbed to human proteins (IB: anti-B2) on enriched membranes of *in vivo* expanded and replated HEK cells transfected with a control RNAi duplex (Cont-RNAi; lane 1) or RNAi duplexes targeting the coding sequence of calreticulin (RNAi-Calret.1/2; lanes 2,3). Upon down regulation of calreticulin, protein levels of the immature B₂ receptor form of ∼53 kDa (immat. B2) were strongly increased. (b) Similar results as in (a) were obtained under *in vivo* conditions with two different, NOD.Scid-expanded HEK-B2 cell clones expressing a control micro-RNA (Cont-RNAi; lanes 1,3), or a micro-RNA targeting the coding sequence of calreticulin (RNAi-Calret.; lanes 2,4). Immunoblots show detection of calreticulin (IB: anti-Calret.; upper panel-left), beta-actin (IB: anti-Actin; lower panel-left), and the B₂ receptor (IB: anti-B2; right panel).
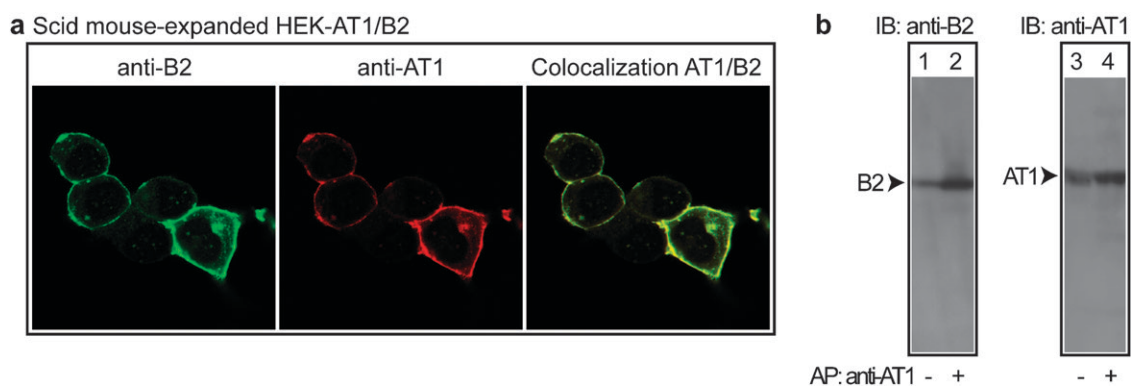
Fig. 5 *In vivo* expansion of HEK293 cells in NOD.Scid mice enhances AT₁/B₂ receptor heterodimerization. (a) Immunofluorescence detection of the B₂ receptor with rabbit anti-B₂ receptor antibodies and Alexa Fluor 488-labeled secondary antibodies (left panel; anti-B2), and AT₁ receptor with rat anti-AT₁ receptor antibodies and Alexa Fluor 546-labeled secondary antibodies (middle panel; anti-AT1) revealed co-localization of AT₁ and B₂ receptors (right panel; Colocalization AT1/B2) on dispersed HEK-AT1/B2 cells isolated after *in vivo* expansion for three weeks in NOD.Scid mice (original magnification, 630×). (b) Immunoblot detection of the B₂ receptor (IB: anti-B2; lanes 1,2), and AT₁ receptor (IB: anti-AT1; lanes 3,4) was performed with solubilized AT₁/B₂ receptor expressing HEK-AT1/B2 cells expanded for three weeks in NOD.Scid mice (lanes 1,3). In lane 2, the B₂ receptor co-enriched with AT₁ receptor-specific antibodies (±AP: anti-AT1) was detected in immunoblot with F(ab)₂ fragments of affinity-purified anti-B₂ receptor antibodies pre-absorbed to human proteins. As a control, the immunoaffinity-enriched AT₁ receptor was detected in immunoblot with anti-AT₁ antibodies (lane 4).

the novel system enabled the study of protein maturation, heterodimerization and function of an important cardiovascular receptor system with transfected cells under *in vivo* conditions.

Discussion

Hyperactivity of the angiotensin II AT₁ receptor is a common feature of cardiovascular disease.[1] Cellular mechanisms accounting for AT₁ receptor sensitization *in vivo* are therefore of major interest regarding the pathogenesis and therapeutic concepts of cardiovascular disorders. Heterodimerization of the angiotensin AT₁ receptor with the bradykinin B₂ receptor leads to sensitization of the angiotensin II response in individuals with cardiovascular risk factors.[3–5] Sensitization of the angiotensin II response relies on the formation of covalently stabilized receptor protein complexes, which provide a kinetically favoured platform for interaction with intracellular signalling molecules relative to dissociable receptors.[6,7]
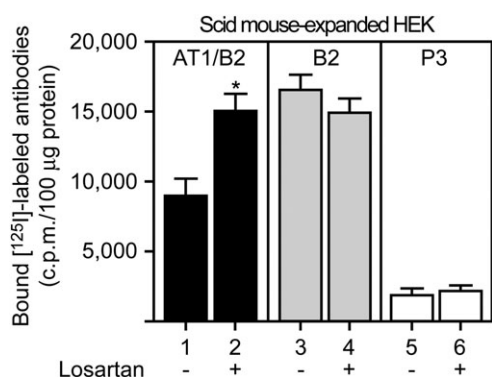
Fig. 6 Assessment of AT_1/B_2 receptor function *in vivo* by drug treatment with the AT_1-specific antagonist losartan. The functional coupling of AT_1/B_2 receptor heterodimers of *in vivo* expanded cells was assessed by the sensitivity to angiotensin II stimulation. *In vivo* stimulation of AT_1/B_2 receptors by circulating angiotensin II was analyzed by B_2 receptor quantification of cells expanded in NOD.Scid mice and treated with or without the AT_1-specific antagonist losartan. B_2 receptors were quantified with $[^{125}I]$-labeled $F(ab)_2$ fragments of affinity-purified anti-B_2 receptor antibodies (1 μCi/point; final concentration 5×10^{-8} M) on dispersed NOD.Scid mouse-expanded HEK cells expressing either AT_1/B_2 (columns 1,2; AT1/B2) or B_2 receptor only (columns 3,4; B2). As a control, the binding assay was performed with *in vivo* expanded HEK-P3 cells without B_2 receptor expression (columns 5,6; P3). HEK cells were isolated after *in vivo* expansion in NOD.Scid mice treated without (−) or with (+) losartan for three weeks (30 mg kg^{-1}) as indicated. Three individual experiments were done in triplicate each. The data represent mean ± S.E., *, $P < 0.03$.

However, factors, which account for the formation of covalently associated AT_1/B_2 receptor heterodimers are still not clear. Several groups showed that heterodimerization of the B_2 receptor with the angiotensin II AT_1 receptor or the closely related AT_2 receptor is very effective in a native environment.[2–6,17] In contrast, under *in vitro* conditions, B_2 receptor maturation and heterodimerization can be severely affected by chosen cell and culture conditions, which may disturb the fine-tuned equilibrium of the general chaperone system.[10,18]

In view of the pathophysiological importance of AT_1/B_2 receptor heterodimers, we sought to establish a cell system, which enables to study that important cardiovascular receptor system in a physiological environment. Cultured human embryonic kidney (HEK) cells were implanted subcutaneously into immunodeficient NOD.Scid mice, and expanded in the well-controlled physiological environment of mice. We chose HEK cells because several scientific facts make human embryonic kidney cells a suitable cell line for the functional study of cardiovascular targets, and specifically the angiotensin II AT_1 receptor. (I) Kidney cell damage is a common feature of many cardiovascular disorders, *e.g.* hypertension, atherosclerosis, diabetes, heart failure.[19–21] (II) Renal cell damage is induced by AT_1 receptor stimulation and excessive angiotensin II release in the course of cardiovascular disease.[22] (III) Microarray gene expression data showed renal epithelial, glomerular, and podocyte markers indicative of the embryonic, renal phenotype of *in vivo* expanded HEK cells. In view of the causal relationship between cardiovascular disease, renal cell

destruction and the angiotensin II system as a major player, the human embryonic kidney cell (HEK) is capable of revealing important aspects of angiotensin-related effects on cardiovascular disease-induced organ cell damage.

Microarray analysis was used to validate the novel *in vivo* system. Microarray gene expression profiling revealed that the expansion of HEK cells for three weeks *in vivo* in immunodeficient NOD.Scid mice affected only a small subset of chaperones while leaving the general glucose-sensitive chaperone system largely unaltered. Notably, there was a major enhancement of calreticulin expression in NOD.Scid-expanded cells relative to conventional cell culture. Thus, the *in vivo* model seemed to restore an imbalance of the general chaperone system induced by conventional cell culture conditions.

Calreticulin is an indispensable chaperone,[23,24] and maturation of the B_2 receptor protein relies on calreticulin.[10,14] In agreement with the importance of calreticulin for the B_2 receptor protein, the maturation of B_2 was strongly enhanced in the novel system. RNA interference revealed a causal relationship between calreticulin expression and enhanced B_2 receptor maturation. Concomitantly to the enhanced protein maturation, interaction of the B_2 receptor with AT_1 was strongly supported in the novel *in vivo* system as demonstrated by immunofluorescence and co-enrichment studies. The AT_1/B_2 receptor heterodimers synthesized in HEK cells under *in vivo* conditions were also functionally active, stimulated and co-internalized by circulating angiotensin II because treatment of NOD.Scid mice with the AT_1-specific antagonist, losartan, substantially increased the number of B_2 receptors on expanded HEK293 cells with AT_1/B_2 heterodimers. Thus, the newly established system enables the study of protein maturation and function under *in vivo* conditions.

In addition to the functional analysis of an important cardiovascular receptor system, our experiments provide strong evidence that the established model is also suitable to study the effect of drug action under *in vivo* conditions in general. During cell expansion, the transplanted cells seem to be effectively connected to the vascular system of NOD.Scid mice. As a consequence, a prototypic cardiovascular drug-such as losartan-supplied in drinking water to NOD.Scid mice could gain access to the proteins of expanded HEK293 cells.

Conclusions

Our study established a novel model to analyze the function of proteins under *in vivo* conditions by applying immunodeficient NOD.Scid mice for *in vivo* expansion of cells. Using the model we studied AT_1/B_2 receptor heterodimers, which is an important cardiovascular receptor system and contributes to an exaggerated angiotensin II response in individuals with cardiovascular disease. With the novel model, we show that efficient B_2 receptor maturation is a prerequisite for heterodimerization with the angiotensin II AT_1 receptor under *in vivo* conditions. Validation of the system by microarray gene expression profiling revealed restoration of an imbalanced chaperone system in the novel *in vivo* cell expansion model relative to conventional cell culture. The model also proved suitable for the study of drug action under *in vivo* conditions as exemplified with a widely used cardiovascular drug, *i.e.* losartan.

Acknowledgements

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