Antiviral effect of Bosentan and Valsartan during coxsackievirus B3 infection of human endothelial cells

Carsten Funke,1 Martin Farr,1 Bianca Werner,1 Sven Dittmann,1 Klaus Überla,2 Cornelia Piper,1 Karsten Niehaus3 and Dieter Horstkotte1

Correspondence
Martin Farr
mfarr@hdz-nrw.de

1Department of Cardiology, Heart and Diabetes Center NRW, Ruhr University of Bochum, Georgstr. 11, 32545 Bad Oeynhausen, NRW, Germany
2Department of Molecular and Medical Virology, Ruhr University of Bochum, Universitätsstr. 150, 44801 Bochum, NRW, Germany
3Center for Biotechnology (CeBiTec), Bielefeld University, Universitätsstr. 27, 33615 Bielefeld, NRW, Germany

In viral myocarditis, adeno- and enteroviruses have most commonly been implicated as causes of infection. Both viruses require the human coxsackie-adenovirus receptor (CAR) to infect the myocardium. Due to its crucial role for viral entry, CAR-downregulation may lead to novel approaches for treatment of viral myocarditis. In this study, we report on pharmaceutical drug influences on CAR levels in human umbilical vein endothelial cells (HUVEC) and cervical carcinoma cells (HeLa) detected by immunoblotting, quantitative real-time PCR and cellular susceptibility to the cardiotropic coxsackie-B3 virus strain Nancy (CVB3). Our results indicate, for the first time, a dose-dependent CAR mRNA and protein downregulation upon Valsartan and Bosentan treatment. Most interestingly, drug-induced CAR diminution significantly reduced the viral load in CVB3-infected HUVEC. In order to assess the regulatory effects of both drugs in detail, we knocked down their protein targets, the G-protein coupled receptors angiotensin-II type-1 receptor (AT1R) and endothelin-1 type-A and -B receptors (ETAR/ETBR) in HUVEC. Receptor-specific gene silencing indicates that CAR gene expression is regulated by agonistic and antagonistic binding to ETBR, but not ETAR. In addition, neither stimulation nor inhibition of AT1R seemed to be involved in CAR gene regulatory processes. Our study indicates that Valsartan and Bosentan protected human endothelial cells from CVB3-infection. Therefore, besides their well-known anti-hypertensive effects these drugs may also protect the myocardium and other tissues from coxsackie- and adenoviral infection.

INTRODUCTION

Viral-induced myocarditis is characterized by inflammatory infiltration of the myocardium, resulting in tissue damage, which in some acute and rapidly progressive cases may result in heart failure, arrhythmias and sudden cardiac death (Abelmann, 1973; Mahrothd et al., 2006). In young adults, myocarditis causes up to 20% of all cases of sudden death (Eckart et al., 2004). As a late consequence of viral-induced myocarditis some patients may develop a dilated cardiomyopathy (DCM). Approximately 30% of patients with DCM present with elevated levels of cardiotoxic viruses like adeno- and enteroviruses (Bowles et al., 1986; Dec et al., 1985). Group B coxsackieviruses (CVB) as well as adenoviruses must cross endothelia to induce infection of the myocardium and both use the coxsackie-adenovirus receptor (CAR) (Carson, 2001). In addition to CAR, coxsackieviruses are also known to use the decay accelerating factor (DAF), whereas adenoviruses use receptors like integrin αVβ3 and 5 as cofactors for viral cell entry (Noutsias et al., 2001). However, in the absence of CAR, neither adeno- nor coxsackieviruses are capable of infecting cardiomyocytes, hence no myocarditis may develop (Shi et al., 2009). CAR is a transmembrane protein, 48 kDa in size, with two extracellular immunoglobulin-like domains located at the N-terminal moiety of the protein, a single membrane-spanning helix with a short cytoplasmic tail (Freimuth et al., 2008).

Tissue distribution of the receptor varies widely in vivo, including heart, lung, brain, liver and kidney. Most notably, CAR seems to be highly upregulated during the
embryonic and early post-natal periods; however, the overall expression level tends to decrease with age. Therefore, particularly high level of CAR expression in the myocardium of young individuals may explain their apparently higher susceptibility to viral myocarditis (Yajima & Knowlton, 2009). Cardiotropic viruses interact with CAR organized in tight junctions at the basolateral side of epithelial cells and subsequent receptor-mediated conformational changes in the virus capsid eventually enables virus entry and release of viral RNA (Freimuth et al., 2008). Due to the crucial role of CAR for viral entry into the myocardium (Kallewaard et al., 2009; Shi et al., 2009), we hypothesized that reduced level of CAR, exposed to the luminal vascular surface and/or myocardial cells, results in a lower susceptibility to viral infection.

The therapeutic potential of CAR ablation, i.e. a cardio-protective effect during viral infection of the myocardium has been discussed (Noutsias et al., 2001). However, there is currently no effective causal therapy of viral myocarditis (Dennert et al., 2008; Pinkert et al., 2009).

Before this background, we analysed various pharmaceutical substances for their gene regulatory effects on the virus receptor CAR in vitro.

RESULTS

Drug-induced effects on CAR mRNA level in human endothelial and epithelial cells

CAR mRNA levels were assessed by quantitative (q) real time (RT)-PCR, following a 24 h incubation with drug doses ranging from 1- to 20-fold of the respective peak plasma concentrations ($c_{\text{max}}$).

Of all the drugs investigated (Supplementary Table S3, available in JGV Online), two revealed a considerable deviation from basal CAR transcription. Administration of Valsartan at concentrations ranging from 1- to 20-fold of $c_{\text{max}}$ reduced CAR mRNA by a maximum of 68.1 ($\pm 8.7$) % in human umbilical vein endothelial cells (HUVEC) and 51.4 ($\pm 8.8$) % in cervical carcinoma cells (HeLa) (Fig. 1a and Supplementary Fig. S1, available in JGV Online).

Bosentan also drastically decreased the CAR mRNA level in HUVEC and HeLa. Maximal reductions by 80.2 ($\pm 4.6$) % in Hela cells and by 66.3 ($\pm 12.6$) % in HUVEC were detected using 10-fold $c_{\text{max}}$ of Bosentan (Fig. 1b and Supplementary Fig. S1). As previously described, induction of CAR gene expression was confirmed by the administration of histone deacetylase inhibitors (Supplementary Fig. S2, available in JGV Online) (Pong et al., 2003).

Western blot analysis of CAR expression in drug-treated cells

HUVEC pretreated for 48 h with fivefold $c_{\text{max}}$ of Bosentan showed a distinct reduction of CAR protein by 44.9 ($\pm 22.7$) % (Fig. 2d). Moreover, cell exposure to 10-fold $c_{\text{max}}$ of Valsartan for 48 h decreased the receptor concentration by 53.8 ($\pm 24.3$) % (Fig. 2b).

Immunofluorescence microscopy

CAR protein expression of vital HUVEC was investigated by immunofluorescence microscopy. The HUVEC monolayer

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![Graph](https://via.placeholder.com/150)

**Fig. 1.** Dose–response curves for either the AT$_1$R antagonist Valsartan (a) or the ET$_A$/ET$_B$R antagonist Bosentan (b) as determined by qRT-PCR in HUVEC after 24 h drug incubation. The detected quantity of CAR mRNA was normalized to that of the housekeeping gene (HKG) $\beta$-actin, and the relative normalized levels are shown ($n=8$, mean $\pm$ SD, **$P<0.01$).
was exposed to Valsartan (10 × c_max) and Bosentan (5 × c_max) for 24 or 48 h (Fig. 3). Both exposures resulted in a considerably reduced FITC (fluorescein isothiocyanate) signal, particularly after the 48 h incubation period, indicating a drug-induced loss of virus receptor on the cellular surface (Fig. 3c, f).

Viral infection of endothelial cells

Finally, we investigated the effect of reduced CAR expression in HUVEC on their susceptibility to viral CVB3 infection. Viral RNA copies were measured by qRT-PCR using viral-specific TaqMan probes.

In comparison to untreated control HUVEC, the viral load of drug-treated cells was significantly lower for both Bosentan and Valsartan at concentrations varying from 1- to 5- and 1- to 10-fold of c_max, respectively (Fig. 4).

Effects of antagonistic and agonistic binding to the endothelin-1 type-A receptor (ETAR)/endothelin-1 type-B receptor (ETBR) on CAR expression in endothelial cells

HUVEC were incubated with Ambrisentan and BQ-788, two selective antagonists for either the ETAR or ETBR (Kingman et al., 2009; Okada & Nishikibe, 2002). Exposure to BQ-788 resulted in a dose-dependent reduction of CAR expression by 26.3 (± 15.0) % (100 nmol l⁻¹) and 43.8 (± 22.1) % (200 nmol l⁻¹), respectively. The administration of Ambrisentan had no influence on the relative CAR mRNA transcript concentration, even at concentrations 10-fold higher than the respective c_max (Fig. 5a).

Furthermore, HUVEC were co-incubated with 2.5-fold c_max of the non-selective ETAR/ETBR antagonist Bosentan and different concentrations of the non-selective agonist endothelin (ET-1), respectively (Fig. 5b). The CAR reducing effects of Bosentan by 53.6 (± 7.3) % were dose dependently attenuated by the addition of 0.01–0.2 μmol ET-1 l⁻¹, resulting in a maximal recovery of CAR to 78.2 (± 18.9) % of its original basal expression level. Further, the exclusive administration of 0.2 μmol ET-1 l⁻¹ resulted in a CAR mRNA accretion of 42.9 (± 28.1) % compared with untreated control cells (Fig. 5b).

RNA interference assays using human endothelial cells

Because Valsartan is an effective competitive antagonist of the AT₁R in vivo and Bosentan in turn serves as a dual antagonist for the ETAR and ETBR, we further investigated the effects on CAR level by silencing all three G-protein coupled receptor genes.

For this purpose, we tested three different siRNAs directed against the ETAR, ETBR and AT₁R genes. Maximal knockdown efficiencies were determined by qRT-PCR and immunoblotting.

This approach resulted in maximal mRNA transcript repressions of ETAR by 91.6 (± 2.5) % and of ETBR by 91.3 (± 4.3) %, respectively (Fig. 6c, f). The AT₁R mRNA
concentration was reduced by 87.6 (± 7.8) % compared with the basal expression level of control cells (Fig. 7c). For this control cell population, a negative-control siRNA sequence was used (Figs 6c, f and 7c).

Furthermore, silencing of all three genes, using 0.04 μmol gene-specific siRNA l21 resulted in an explicit reduction of the respective receptor proteins, 48 h after transfection. Moreover, protein concentration was reduced to almost

Fig. 3. Semi-quantitative immunofluorescence microscopy of CAR expressing HUVEC monolayers pretreated with either Valsartan (b–c) or Bosentan (e–f). Untreated HUVEC served as negative controls (a, d). All cells were cultured for 48 h and analytics started directly after drug incubation. The receptor density was assessed with a polyclonal rabbit anti-CAR antibody and an FITC-labelled secondary antibody (green). DAPI counter staining was used to normalize FITC fluorescence intensities of each cellular monolayer (blue). All experiments were at least performed in quadruplicate and representative microscopic pictures are shown (×200 magnification).

Fig. 4. CVB3 infection of HUVEC pretreated for 48 h with different cmax of Valsartan (a) or Bosentan (b). After incubation with these drugs, cells were inoculated with a 102× TCID50 of CVB3. Post-infection (4 h) viral RNA copies were detected using qRT-PCR and viral-specific TaqMan probes. Infected but otherwise untreated HUVEC (Standard, Std) were used to normalize the CVB3 RNA copy number of each sample. For each qRT-PCR assay, a dilution series of a CVB3 control plasmid DNA (pEV) served as internal reference control. All experiments were performed at least in triplicate and data are expressed as mean ± SD (**P<0.01).
undetectable levels 72 h after transfection (Figs 6b, e and 7b).

To exclude unspecific gene silencing for all conducted knockdowns, Western blot analyses utilizing antibodies against AT1R, ETAR, ETBR and CAR were performed.

The immunoblots of HUVEC treated with gene-specific or unspecific control siRNAs and untreated cells revealed almost no off-target effects, whereas siRNAs targeting AT1R, ETAR and ETBR reduced the corresponding signals from these proteins to almost undetectable levels (Supplementary Fig. S3, available in JGV Online). These results were confirmed by qRT-PCR assays using gene-specific DNA probes for all four receptor types (data not shown).

**Drug effects on CAR expression in ETAR- and ETBR-repressed HUVEC**

To study functional consequences of ETAR and ETBR knockdown in HUVEC, these cells were exposed to previously applied concentrations of receptor-specific agonists and antagonists 72 h after gene-specific siRNA or control-siRNA transfection (Fig. 6a, d). Thus, for an additional period of 24 h, the ETAR- or ETBR-repressed HUVEC were respectively incubated with Ambrisentan, BQ-788, Bosentan and ET-1.

ETAR knockdown itself, without the influence of any drugs, did not significantly alter CAR mRNA levels compared to untreated and control-siRNA transfected cells (Fig. 6a). Moreover, the drug induced influence on CAR mRNA expression remained almost constant in the ETAR-repressed cells. Thus, as shown earlier without the previous application of RNA interference (Fig. 5b), ET-1 administration at a concentration of 0.2 μmol l⁻¹ induced CAR mRNA expression by 48.3 (±10.1)% (Fig. 6b). Furthermore, a 2.5-fold $c_{max}$ of Bosentan reduced CAR mRNA expression by 47.5 (±16.9)%.

siRNA-induced repression of the ETBR diminished CAR mRNA concentration by 31.4 (±2.5)% relative to the untreated control cell population. Neither the administered antagonists nor the agonist ET-1 had further influence on the expression level of CAR, which remained reduced by approximately 30–40% in all ETBR-repressed HUVEC (Fig. 6d).

**Drug effects on CAR expression in AT1R-repressed HUVEC**

CAR expression was detected after siRNA-mediated down-regulation of AT1R and subsequent drug administration, as previously described for ETAR/ETBR-reduced cells.

The receptor downregulation did not provoke any significant changes in CAR mRNA quantity compared to untreated and control cells pretreated with unspecific siRNA (Fig. 7a). Administration of the AT1R agonist angiotensin-II at high concentration (1 μmol l⁻¹), also did not effect CAR expression in either AT1R-deficient or -positive HUVEC. In turn, cells responded to Valsartan administration at a concentration 10-fold higher than $c_{max}$ and CAR mRNA was reduced by 63.1 (±19.1)% in AT1R-repressed HUVEC (Fig. 7a). In comparison, equally treated but AT1R-positive HUVEC showed similar CAR mRNA reduction by 59.6 (±4.9)% (Fig. 1a).
DISCUSSION

Current therapeutic options in viral myocarditis are limited (Bowles et al., 1986). Probably more than 50% of virus-related myocardial inflammation is caused by adenoviruses and enteroviruses. Hence, the therapeutic potential of CAR ablation during viral infection of the myocardium has been...
discussed previously (Freimuth et al., 2008; Noutsias et al., 2001). In the present study, we demonstrate a down-regulatory effect of the 7-transmembrane G-protein coupled receptor antagonists Bosentan and Valsartan on CAR gene expression in human endothelial and epithelial cells (Figs 1–5).

qRT-PCR using CAR-specific TaqMan probes indicates that the mRNA expression in both HeLa and HUVEC correlates inversely on the concentration of administered drug (Fig. 1 and Supplementary Fig. S1).

In previous studies, human primary vascular cells were exposed to 10\,\mu\text{mol} Bosentan l^{-1} and 100\,\mu\text{mol} Valsartan l^{-1}, which corresponds to 14- and 22-fold of \(c_{\text{max}}\), respectively (Bian et al., 2009; Harada et al., 1997). In our study, Bosentan and Valsartan at concentrations ranging from 0.5 to 10-fold of \(c_{\text{max}}\) reduced CAR mRNA in HUVEC by a maximum of 66.3 (\(\pm 12.6\)) and 68.1 (\(\pm 8.7\)) %, respectively. Corresponding drug exposure to HeLa cells revealed a maximal CAR decrease by either 80.2 (\(\pm 4.6\)) % for Bosentan or 51.4 (\(\pm 8.8\)) % for Valsartan (Supplementary Fig. S1).

In good agreement with the mRNA expression data, the CAR protein detected via immunoblotting was considerably reduced as well. Herein, CAR levels were maximally decreased by 53.8 (\(\pm 24.3\)) and 44.9 (\(\pm 22.7\)) % in HUVEC after 48 h exposure to Valsartan (10\,\times\,c_{\text{max}}) and Bosentan (5\,\times\,c_{\text{max}}), respectively (Fig. 2b, d). Furthermore, membrane-bound CAR exposed to the extracellular space of vital HUVEC monolayer was quantified by immunofluorescence microscopy using CAR-specific and FITC-labelled antibodies. Exposure of HUVEC to either Bosentan (5\,\times\,c_{\text{max}}) or Valsartan (10\,\times\,c_{\text{max}}) for 24 and 48 h resulted in a considerable reduction of CAR expression (Fig. 3).

Summarizing the qRT-PCR and relative protein quantification assays, our findings indicate that not only the mRNA and total protein amount, but also the receptor density at the surface of the endothelial cells was drastically reduced by both the dual ET-1 receptor antagonist Bosentan and the AT\(_1\)R antagonist Valsartan. Most notably the receptor reduction was detected as a function of drug concentration and incubation time. These results evidently suggest signal transduction effects are attributable to the antagonistic binding of both compounds to their respective receptor. To study whether both ET-1 receptor subtypes are involved in Bosentan induced decline of CAR expression \textit{in vitro}, endothelial cells were treated with dual and selective antagonists and agonists of both receptor subtypes and the relative CAR mRNA expression was detected in these cells (Fig. 5).

The administration of BQ-788, a highly selective ET\(_A\)R antagonist (Ishikawa et al., 1994) resulted in a dose-
dependent reduction of CAR expression to a maximum of 56.2 (± 22.1) % of its original level, whereas administration of the selective ET₄R antagonist Ambrisentan (Barst, 2007) had no effect on the relative CAR mRNA transcript concentration, even at concentrations 10× higher than the respective cₘₐₓ (Fig. 5a). These data lead to the assumption that transcriptional CAR regulation in HUVEC is controlled by ET₄R, but not ET₃R signalling.

To further consolidate these findings, the experiments were iterated, this time with prior siRNA induced knockdown of either the ET₄R or ET₃R. Therefore, an optimum of gene silencing efficiency was confirmed by qRT-PCR and immunoblotting 72 h after 0.04 μmol l⁻¹ siRNA transfection (Fig. 6). The receptor repressed cells were subsequently exposed to previously applied dual and selective antagonists and agonists of both receptor subtypes and the relative CAR expression was detected in these cells. Thus, we demonstrated that siRNA-mediated specific repression of ET₄R and subsequent Bosentan treatment results in the same downregulation of CAR as determined without any siRNA exertion (Figs 1b and 6a). Furthermore, exogenous ET-1 augments CAR mRNA level equally effectively in both ET₄R-positive and repressed cells (Figs 5b and 6a).

On the other hand, treatment with the same drugs and knockdown of ET₄R caused almost no alteration of CAR transcript concentration compared to untreated control cells (Fig. 6d). In addition, the ET₄R-specific antagonist BQ-788 also seems to reduce CAR level in a dose-dependent manner (Fig. 5a).

In conclusion, these findings further strengthen the assumption that CAR gene expression seems to be regulated by both, stimulant as well as antagonistic binding to the ET₄R, but not ET₃R. CAR downregulation attributable to ET₄R signalling is supported by previous studies, suggesting that administration of 17β-oestradiol – known to downregulate ET₄R in the heart – during CVB3-induced myocarditis in mice results in significant alleviation of viral infection (Huber et al., 1999; Nuedling et al., 2003).

To figure out whether the regulatory mechanism of Valsartan is caused by antagonistic binding to the corresponding AT₄R, this receptor was knocked down similarly to ET₄R and ET₃R (Figs 6 and 7). As a result, the receptor downregulation did not provoke any effects of Valsartan on CAR mRNA quantity. Furthermore, exogenous stimulus of the AT₄R by angiotensin-II, the receptor’s natural agonist, did not induce any changes of CAR transcript levels (Fig. 7a). These findings indicate that the CAR repressing effect of Valsartan is obviously not mediated through the AT₄R. Complementing these findings, none of the other administered Sartans (drug family of AT₄R antagonists), like Telmisartan, Candesartan (CV-11974), Losartan, etc. revealed any effects on the expression level of CAR in HUVEC (data not shown), again indicating an AT₄R-independent gene regulatory mechanism not yet described.

Since CAR is well known as an essential cofactor for viral binding and cellular entry, in its absence adenov- and coxsackieviruses do not infect the myocardium (Freimuth et al., 2008; Kallewaard et al., 2009; Shi et al., 2009). In order to figure out whether the drug induced decrement of CAR exposed to the cell surface of HUVEC alters their susceptibility to viral infection, these cells were infected with CVB3 (Fig. 4).

The infection studies revealed a significant lowering of viral RNA copy numbers isolated from drug-treated cells in comparison to untreated control cells, indicating a virus protective dose-dependent effect of Bosentan and Valsartan in cultured endothelial cells (Figs 1–4).

The most impressive reduction of viral RNA copy number by approximately 70–90 % was detected in HUVEC pretreated with 5× cₘₐₓ Bosentan or 10× cₘₐₓ Valsartan (Fig. 4). Considering the comparatively moderate reduction of CAR mRNA and protein level in similarly treated cells by not more than approximately 60 % (Figs 1 and 2), the question remains whether the antiviral effects of both compounds are explainable by reduction of the virus receptor alone. Besides CAR as primary receptor for all serotypes of CVB (Martino et al., 2000), the existence of several other factors crucial for viral permissivity has been reported (Johansson et al., 2004; Zautner et al., 2003). In particular, the CVB3 requires several additional proteins for effective adhesion and subsequent integration, as demonstrated by the non-permissivity of DAF-, occludin- and/or dynamin-deficient cells (Coyne et al., 2007; Patel et al., 2009). While certainly interesting, none of these influences were addressed by our study and therefore we can only hypothesize about the impact of these potential mediators of CVB3 infection on our results, so far.

Although, it remains to be clarified whether CAR reduction is either exclusively or rather partially responsible for the drug triggered antiviral effects, our study clearly illustrates that Valsartan and especially Bosentan show significant cell-protective features, even with low dosages. These findings become particularly relevant when considering the clinical potential of both drugs in the treatment of virus myocarditis. Thus, our CVB3 infection studies revealed a remarkable reduction of viral copy numbers by more than 60% even at physiological concentrations of 1× cₘₐₓ Bosentan (Fig. 4b). Nevertheless, these in vitro results have to be reproduced in vivo with focus on pharmacokinetics and the impact of CAR-independent antiviral effects triggered by both therapeutic drugs.

In general, the role of CAR as the primary virus receptor for all CVB serotypes and some adenoviruses remains uncontroversial, as indicated by several studies assessing the impact of CAR reduction on cardiotoxic infections. Thus, Kallewaard et al. (2009) recently demonstrated that cardiac-specific deletion of CAR protects mice from CVB-induced myocarditis by significantly reducing virus levels and virus-induced tissue damage. Shi et al. (2009) further demonstrated that CAR elimination in murine hearts
efficiently blocks CVB3 entry and therefore prevents the development of myocarditis.

Moreover, beneficial effects of soluble CAR isoforms on cardiac CVB3 infections have been reported previously (Lim et al., 2006; Pinkert et al., 2009; Yanagawa et al., 2004), emphasizing the contribution of CAR-mediated pathology in viral myocarditis.

In a murine model of viral myocarditis, Ono et al. (1999) showed that ET-1 signalling plays an exacerbating role and that treatment with Bosentan otherwise has a cardioprotective effect. Marchant et al. (2009) demonstrated beneficial effects on cardiac function in Bosentan-treated mice, but otherwise an increase in copy number of viral genomes via ETAR-mediated p38 mitogen-activated protein kinase (MAPK) activation.

Because in these experiments Bosentan treatment started after an initial infection with CVB3, the increasing number of viral genomes in murine heart sections might reflect p38 MAPK-mediated virus replication within initially infected cells rather than viral spread within formerly unaffected myocardium. As viral infection is a multifocal process (Kandolf et al., 1987) not only the overall reduction of viral copy number (mainly influenced by replication) within infected cells may be relevant for progression of infection, but also the prevention of ongoing cell-to-cell infection (viral uptake). Hence, it has to be clarified which of these effects (either the p38 MAPK triggered enhancement of viral replication or the prevention of de novo cell infection by reduction of the primary virus receptor) predominates during infectious myocarditis. Therefore, we agree with Marchant et al. (2009) by proposing a long-term assessment of the pharmacological intervention with Bosentan, not only in the murine model, but also in human in vitro assays. Hence, it has to be clarified whether administration of Bosentan will sufficiently limit an acute viral myocarditis and/or prevent a chronic course of the disease.

As previously mentioned, the beneficial effect of cardiac-specific CAR ablation during experimental CVB3-induced myocarditis has been recently reported by various investigators (Kallewaard et al., 2009; Shi et al., 2009). Furthermore, detrimental effects of CAR knockout in vivo were reported by Lisewski et al. (2008).

Nonetheless, it is important to note that these investigations focused on the influence of a complete depletion of CAR in vivo. Yet, it can be assumed that a pharmacological treatment, as for instance with Bosentan and Valsartan, would probably not result in a complete depletion of CAR protein, but rather in a certain reduction as shown in Figs 1–4. Even if complete knockout of CAR might induce side effects, as reported by Lisewski et al. (2008), there is still no compelling evidence that pharmacologically triggered CAR reduction would cause similar side effects.

However, future attempts to figure out the potential use of both Bosentan and Valsartan as antivirals should also take into consideration that inhibition of CAR might cause unwanted side effects.

In summary, the present study demonstrates, for the first time, a downregulatory effect on CAR gene expression by Bosentan and Valsartan, two long-term approved cardiovascular drugs. Expression decline was detected at protein and mRNA levels and – in case of Bosentan – the receptor decrease was attributable to an ETAR-dependent signalling mechanism (Fig. 8). In comparison, the pathway through which Valsartan effects CAR gene expression seems to be AT1R independent and remains to be elucidated.

Most notably, we could demonstrate that CVB3-infection rates were significantly reduced in endothelial cells after exposure to Bosentan and Valsartan. Subsequent experiments with other CAR-binding viruses like adenoviruses AdV2 and AdV5 (Tomko et al., 1997) or different serotypes of CVB (Martino et al., 2000) as well as experiments with other human cell types, e.g. cardiomyocytes have to prove our concept of antiviral downregulation of CAR.

METHODS

Cell culture. Cell culture conditions were 37 °C in a 100 % humidified 5 % CO2 atmosphere. HUVEC were cultured in Endothelial Cell Growth Medium-2 (Lonza) on collagen-coated surfaces.

HeLa were cultured in RPMI 1640 medium (PAA Laboratories) supplemented with 1 % penicillin/streptomycin and 10 % fetal bovine serum.

Drug screening. The applied drugs and chemicals include several substance classes. For a list of all substances, their peak plasma concentrations (cmax) and respective clinical trials, please refer to Supplementary Table S3. Cell cultures were incubated for 24–72 h with these compounds at different concentrations, varying from 0.5 to 20-fold of their respective cmax.

For an exemplary comparison of mRNA and protein levels, the respective drug concentrations used for immunocytochemical and densitometric evaluation of relative CAR expression were chosen on the basis of previously received dose–response curves by qRT-PCR (Fig. 1).

Since cytotoxicity of Valsartan and Bosentan may affect CAR expression as well as viral replication, measurements of cell viability and cytotoxicity of these drugs were performed prior to the qRT-PCR and infection experiments in HUVEC and HeLa. Therefore, different concentrations of both substances varying from 1- to 100-fold of the respective cmax were tested for 72 h (including a medium and drug change every 24 h). The cell viability was confirmed by microscopic assessment of changes in cell morphology and confluence as well as vital cell counting using trypan blue staining. As a result, even drug concentrations 100-fold higher than cmax of Bosentan (Valsartan) revealed no cytotoxic effect and were tolerated by both cell types (data not shown).

Immunocytochemistry. HUVEC were subcultured on 0.8 cm2 glass surface areas, washed twice with PBS, then fixed with ethanol (15 min at 4 °C) and subsequently incubated at room temperature for 60 min in blocking solution comprising 0.2 % BSA in PBS. After washing with PBS, CAR was stained with polyclonal rabbit anti-CAR antibody (Supplementary Table S2, available in JGV Online) for 4 h in PBS containing 0.2 % BSA. After washing, the membrane was stained with Alexa Fluor 555-conjugated donkey anti-rabbit secondary antibody (Invitrogen) and counterstained with DAPI (Invitrogen) for 1 h in dark. After washing, slides were mounted with Fluoromount-G (Southern Biotechnology Associates).
blocking solution at room temperature. After two washing steps with PBS, the primary antibody was detected with an FITC-conjugated polyclonal mouse anti-rabbit-IgG (Supplementary Table S2, available in JGV Online) in blocking solution for 2 h at room temperature. Exclusive application of the secondary antibody was used as control. Cells were rinsed twice with PBS, and DAPI (4,6-diamidino-2-phenylindole) at 0.5 \( \mu \text{gml}^{-1} \) for 5 min at room temperature was used to label cell nuclei. After three additional washing steps with PBS, the slides were directly investigated under the fluorescence microscope equipped with FITC (485 nm) and DAPI (365 nm) filters. DAPI signal intensities were normalized and used for relative quantification of the respective FITC signal.

Western blot analysis. Cells were washed twice with PBS and lysed in homogenization buffer (50 mmol Tris/HCl \( \text{L}^{-1} \), pH 8.0, 2.5% \( \beta \)-mercaptoethanol, 2% sulfobetaine-10) by five cycles of freeze–thaw. After centrifugation at 8000 \( \text{g} \) for 5 min, supernatants were collected and the protein contents of all samples were determined by a modified BCA assay (Smith et al., 1985).

Gel electrophoresis was performed on 4–12% NuPAGE Novex Bis-Tris Gels, loading equal amounts of total protein resuspended in Laemmli buffer (Laemmli, 1970). Transfer to a 0.2 \( \mu \text{m} \) PVDF membrane was followed by blocking in 5% non-fat dried milk in TBST (10 mmol Tris/HCl \( \text{L}^{-1} \), pH 7.5, 150 mmol NaCl \( \text{L}^{-1} \), 0.05% Tween-20). Subsequently, membranes were incubated with primary antibody against CAR, ETAR, ETBR, AT1R or GAPDH (glyceraldehyde 3-phosphate dehydrogenase) for 4 h (Supplementary Table S2). Detection was performed by the use of secondary alkaline phosphatase- or horseradish peroxidase-conjugated antibodies and application of the respective substrate solution according to the supplier’s instructions (BCIP/NBT or ChemiGlow solution; Supplementary Table S2).

All steps were performed at room temperature, and each antibody incubation was followed by three washing steps with TBST buffer for 10 min. Monoclonal mouse anti-GAPDH antibody served as an internal loading control.

The amount of protein was quantified by densitometry using the MultiImager CCD camera system from AlphaInnotech Inc. with the attached ‘Alpha Easy’ software v5.5 and 2D scans of membrane bands (Milting et al., 2006).

BCA assay for protein quantification. Protein samples were diluted (1:20) for the BCA protein assays. Serial dilutions of a BSA protein standard from 0.02 to 2.0 mg ml\(^{-1}\) were used to create a standard absorbance curve. Equal volumes of diluted protein samples and assay solution (pH 11.3, 3.5 mmol sodium bicinchoninate \text{L}^{-1}, 7.5 mmol sodium carbonate \text{L}^{-1}, 0.15 mol sodium tartrate \text{L}^{-1}, 1.5 mmol cupric sulfate \text{L}^{-1}) were combined and subsequently the colorimetric detection was performed using a 96-well plate spectrophotometer (Alpha Innotech) at 562 nm.

Reverse transcription. After RNA isolation (RNasy Mini kit; Qiagen) 450 ng total RNA was transcribed with the Invitrogen SuperScript III Reverse Transcriptase in accordance with the supplier’s protocol.

qRT-PCR. The relative gene expressions of CXADR (CAR), AGT1R (AT1R), EDNRA (ETAR), EDNRB (ETBR), GAPDH, ACTB (\( \beta \)-actin) and RPL32 were assessed by detection of mRNA levels using TaqMan.
probes combined with the Applied Biosystems StepOnePlus-RT-PCR System. ACTB, GAPDH and RPL32 probes were used as internal controls.

The comparative C_{t}-method (ΔΔC_{t}-method) was used for all target genes and was previously validated for comparable PCR efficiencies between targets and endogenous controls according to the supplier’s guidelines (Applied Biosystems).

**Viral infection.** HUVEC were infected with the genetically characterized cardiovirulent Nancy strain of CVB3 (Klump et al., 1990). Therefore, the cells were inoculated with a 10^7 × tissue culture infectious dose-50 (TCID_{50}) of CVB3 and after an incubation period of 4 h at 37 °C, viral RNA was isolated (QiAamp Viral RNA Mini kit; Qiagen) and quantified by qRT-PCR (QuantiTect Probe RT-PCR kit; Qiagen) using CVB3-specific TaqMan probes and primers (Supplementary Table S1). Infected but otherwise untreated HUVEC were used to normalize the CVB3 RNA copy number of each sample. For each qRT-PCR assay, a dilution series of a CVB3 control plasmid DNA (pEV) served as internal reference. The 4 h time point was chosen, since control experiments revealed that there is an approximate 10-fold increase in CVB3 RNA between 2 and 4 h. Reduction of this increase by drug treatment indicates inhibition of an early step during a single viral replication cycle. Thus, after 4 h the changes in viral RNA amount represent the number of de novo infected cells and do not essentially take into account viral replication cycles. Since CAR (as primary virus receptor for CVB3) is responsible for the de novo infection of host cells, and has no influence on viral replication cycles or lytic proliferation, our experiments were particularly designed for quantifying viral load briefly after infection (Bergelson et al., 1997).

**Gene silencing.** siRNAs targeting the human genes AGT1R (AT1R-siRNA: 5'-GUCUCAGGCAU UGAUCGAUAttt-3'), EDNRA (ETAR-siRNA: 5'-GCUUGCUAUGGUACCUCUUt-3') and EDNRB (ETBR-siRNA: 5'-CGUUGGUAUUGGACUAUAtt-3') were confirmed to have knockdown efficiency by qRT-PCR and immunoblotting.

To exclude non-specific gene inhibition, a silencing negative-control siRNA blend was used (AM4611; Ambion). Cells were transfected with different concentrations of siRNA (0.01, 0.04 and 0.08 μmol L^{-1}) with Lipofectamine 2000. The siRNA-mediated downregulation of target genes was assessed 24 and 48 h after transfection by qRT-PCR as well as 48 and 72 h after transfection by protein analysis, respectively.

**Statistics.** All results of this study are expressed as mean values (±SD). The statistics and 2D-graphing software GraphPad Prism v4.3 (GraphPad Software, Inc.) were used for statistical analysis. Differences between groups of data were either assessed by the Student’s t-test or the one-way analysis of variance with Dunnet’s post-hoc test, according to requirements. \( P<0.05 \) was considered significant.

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