Regulation of cardiotrophin-1 expression in mouse embryonic stem cells by HIF-1α and intracellular reactive oxygen species

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Summary

Cardiomyogenesis in differentiating mouse embryonic stem (ES) cells is promoted by cardiotrophin-1 (CT-1), a member of the IL-6 interleukin superfamily that acts through the tall gp130 cytokine receptor. We show that prooxidants (menadione, hydrogen peroxide) as well as chemical ($CoCl_2$) and physiological (1% O_2) hypoxia increased CT-1 as well as HIF-1α protein and mRNA expression in embryoid bodies, indicating that CT-1 expression is regulated by reactive oxygen species (ROS) and hypoxia. Treatment with either prooxidants or chemical hypoxia increased gp130 phosphorylation and protein expression of NADPH oxidase subunits p22-phox, p47-phox, p67-phox, as well as *Nox1* and *Nox4* mRNA. Consequently, inhibition of NADPH oxidase activity by diphenylen iodonium chloride (DPI) and apocynin abolished prooxidant- and chemical hypoxia-induced upregulation of CT-1. Prooxidants and chemical hypoxia activated ERK1,2, JNK and p38 as well as PI3-kinase. The prooxidant- and $CoCl_2$-mediated upregulation of CT-1 was significantly inhibited in the presence of the ERK1,2 antagonist UO126, the JNK antagonist SP600125, the p38 antagonist SKF86002, the PI3-kinase antagonist LY294002, the Jak-2 antagonist AG490 as well as in the presence of free radical scavengers. Moreover, developing embryoid bodies derived from HIF-1α^{−/−} ES cells lack cardiomyogenesis, and prooxidants as well as chemical hypoxia failed to upregulate CT-1 expression. Our results demonstrate that CT-1 expression in ES cells is regulated by ROS and HIF-1α and imply a crucial role of CT-1 in the survival and proliferation of ES-cell-derived cardiac cells.

Key words: Embryonic stem cells, cardiotrophin-1, reactive oxygen species, hypoxia-inducible factor-1, cardiomyogenesis, embryoid body

Introduction

The cytokine cardiotrophin-1 (CT-1) is a member of the IL-6 family of cytokines acting through the heterodimeric complex LIFRβ:gp130 that becomes tyrosine phosphorylated by Janus kinases (Jaks). The Jaks activate multiple downstream signalling pathways, which involve signal transducers and activators of transcription (STATs), MAPKs, PI3-kinase and NF-κB (Freed et al., 2005; Pennica et al., 1996). Several biological effects with vital importance on heart development and function have been attributed to CT-1. In embryonic and neonatal cardiomyocytes CT-1 has been demonstrated to mediate cell proliferation and support cell survival (Sheng et al., 1996). In ES cells CT-1 has been recently shown to stimulate cardiomyogenic differentiation and cell proliferation by a mechanism involving ROS as signalling molecules in Jak/STAT-, MAPK- and NF-κB-mediated signal transduction cascades (Sauer et al., 2004). Besides its important function in embryonic and neonatal tissues CT-1 exerts a protective function in the adult heart by inducing cell hypertrophy through stimulation of sarcomere assembly in series with the subsequent increase in cardiomyocyte cell length (Wollert et al., 1996), and by preventing apoptosis in vitro and in vivo (Sheng et al., 1997). Furthermore, CT-1 has been shown to

stimulate cardiac fibroblast proliferation and migration, thereby allowing repopulation of scars developing after cardiac infarction with cells that promote wound healing and preserve cardiac function (Freed et al., 2003).

So far, the molecular regulation of CT-1 expression is unknown. The properties of CT-1 as a cardioprotective cytokine in stress conditions predict upregulation during cardiac diseases that are characterized by an environment of hypoxia, inflammation and oxidative stress. It could be hypothesized that stress stimuli occurring during cardiac diseases regulate the expression of CT-1, which subsequently exerts its cardioprotective effect. In this respect it has been demonstrated that CT-1 is cardioprotective for ischemia-reperfusion injury, even when added late during reoxygenation (Brar et al., 2001; Liao et al., 2002), and it was discussed that early expression of CT-1 in the ischemic myocardium may represent an adaptive, protective phenomenon that is beneficial in reducing myocyte loss and inducing hypertrophy of remaining myocytes, thereby allowing the maintenance of overall ventricular function (Freed et al., 2005). Indeed elevated serum levels of CT-1 have been observed in patients with unstable angina pectoris (Talwar et al., 2000a), acute myocardial infarction (Freed et al., 2003; Talwar et al., 2000b)

and heart failure (Talwar et al., 2000b). A comparable microenvironment of hypoxia and elevated ROS generation may prevail in the heart of the early postimplantation embryo and could regulate CT-1 expression in the embryonic heart. Hypoxia and robust endogenous ROS production have been previously shown to occur in differentiating ES cells and may represent one key stimulus for regulation of CT-1 expression as well as induction of the cardiomyogenic cell lineage (Sauer et al., 2000).

The current study was performed to evaluate the impact of the stress factors hypoxia and ROS on the signalling cascades resulting in CT-1 expression. Since differentiating ES cells within embryoid bodies mimic cardiomyogenic differentiation (Desbaillets et al., 2000), and knowing that vascularized embryoid bodies are well oxygenated (Gassmann et al., 1996), we made use of this cell culture model. Our data indicate that hypoxia as well as ROS use a common HIF-1-regulated signalling pathway that results in increased CT-1 expression. HIF-1 is a heterodimer composed of the constitutively expressed β-subunit and the oxygen-dependent α-subunit (HIF-1α) that is stabilized under hypoxic conditions (reviewed by Hopfl et al., 2004). Comparable situations of hypoxia and oxidative stress occur in various stress conditions and presumably mediate cardioprotection as well as cardiac cell proliferation and hypertrophy by CT-1 in the embryonic and adult heart.

Results

Increasing CT-1 and gp130 expression in differentiating wt ES cells

We recently showed that treatment of ES-cell-derived embryoid bodies with CT-1 results in stimulation of cardiomyogenesis, and that CT-1 is endogenously expressed in embryoid bodies (Sauer et al., 2004). To correlate CT-1 with the time course of embryoid body differentiation and expression of the transducing receptor gp130, protein levels of CT-1 and gp130 were monitored. Expression of CT-1 ($n=6$) and gp130 ($n=4$) followed a similar expression pattern until day 8 in culture and subsequent downregulation on day 10 and day 11 (Fig. 1A,B). Expression of CT-1 and gp130 culminated at 175±25% and 188±6%, respectively, on day 8 compared with day 2 (set to 100%). It has been previously shown that cardiomyogenic differentiation of mouse ES cells occurs between day 6 and 8, i.e. during the time of maximal CT-1 and gp130 expression (Hescheler et al., 2002).

Regulation of CT-1 and HIF-1α expression by vitamin E

We have previously shown that differentiating embryoid bodies express HIF-1α (Bichet et al., 1999) and robustly generate ROS that may be used as signalling molecules regulating CT-1 expression (Sauer et al., 2000). To support this assumption, embryoid bodies were treated with the free radical scavenger vitamin E (20 μM) from day 2 to day 8 (Fig. 2). Subsequently, CT-1 expression was assessed in untreated and vitamin-E-treated embryoid bodies. Since CT-1 has been previously shown to be upregulated by hypoxic stress in cardiac myocytes (Hishinuma et al., 1999) the expression of HIF-1α was
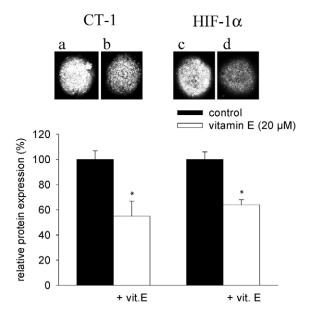

Fig. 1. Protein expression of CT-1 (A) and gp130 (B) during of ES cell differentiation. Determination was performed within the three-dimensional tissue of embryoid bodies. Note that cardiac cell differentiation occurs during day 6 and 8 of embryoid body cell culture (Hescheler et al., 2002). *$P<0.05$, significantly different to levels at day 1 and day 2 of cell culture in A and B, respectively.

Fig. 2. Regulation of CT-1 and HIF-1α expression in embryoid bodies by endogenous ROS. Embryoid bodies were incubated from day 2 to day 8 of cell culture with 20 μM vitamin E. On day 8 protein expression of CT-1 and HIF-1α was assessed by semiquantitative immunohistochemistry in whole-mount embryoid bodies. The images show representative embryoid bodies labeled with antibodies either against CT-1 or HIF-1α which remained untreated (a,c) or were treated with vitamin E (b,d). *$P<0.05$, significantly different to levels in the untreated control.

assessed in parallel to investigate possible correlation with CT-1 expression. It was apparent that vitamin E treatment downregulated CT-1 as well as HIF-1α expression in 8-day-old embryoid bodies (untreated control set to 100%), indicating regulation by ROS endogenously generated in differentiating ES cells (n=4). Assessment of the effects of vitamin E following 24 hours and 72 hours of incubation revealed that HIF-1α and CT-1 were downregulated in parallel (data not shown).

Regulation of CT-1 and HIF-1α expression by exogenous prooxidants

The data above suggest that regulation of CT-1 and HIF-1α occurs by endogenous generation of ROS during differentiation. To validate these findings, 4-day-old embryoid bodies were treated with either the prooxidants menadione or H_2O_2 (Fig. 3A,B). Treatment of embryoid bodies with H_2O_2 in concentrations ranging from 1 nM to 100 μM resulted in the dose-dependent increase of CT-1 protein expression (data not shown). Maximum effects were achieved at 10 μM H_2O_2, which increased CT-1 protein (Fig. 3A) and mRNA expression

(Fig. 3B) to 145±8% and 140±9%, respectively (n=3) (untreated control set to 100%). Likewise, treatment with 10 μM (data not shown) and 20 μM menadione resulted in increased CT-1 expression with maximum values of 157±8 % and 175±7% for CT-1 protein (n=3) (Fig. 3A) and mRNA (n=3) (Fig. 3B) expression, respectively, at 20 μM menadione concentration. Under the same experimental conditions increased protein as well as mRNA expression of HIF-1α was observed. HIF-1α protein expression amounted to 140±8% and 184±9% (see Fig. 3A), and mRNA expression to 220±24% and 264±7% for H_2O_2 and menadione, respectively (see Fig. 3B) (n=5), thus corroborating the notion of parallel regulation of CT-1 and HIF-1α by prooxidants.

Upregulation of CT-1 and HIF-1α expression by hypoxia

Regulation of CT-1 on the protein as well as mRNA level paralleled the regulation of HIF-1α. Consequently, it should be hypothesized that hypoxia results in the reverse situation: upregulation of CT-1. To address this assumption, 4-day-old embryoid bodies were exposed to either 24 hours of physiological (1% O_2) or chemical (50 μM $CoCl_2$) hypoxia
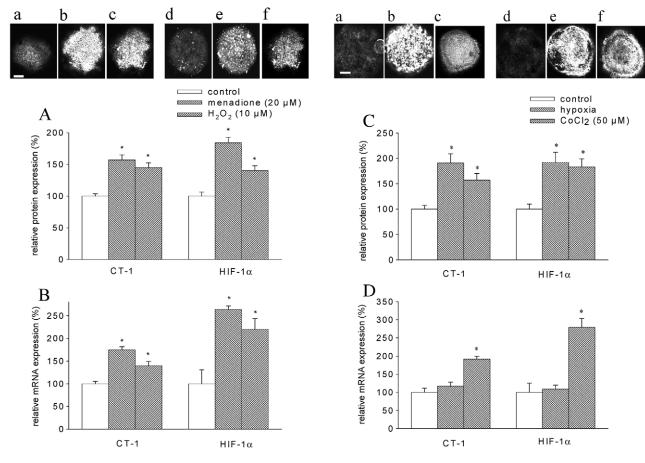

Fig. 3. Upregulation of CT-1 and HIF-1α protein and mRNA upon treatment with prooxidants and hypoxia. (A,B) Embryoid bodies were treated on day 4 with menadione (20 μM) and H_2O_2 (10 μM). Protein (A) and mRNA (B) levels were examined 24 hours later. The images above A show representative embryoid bodies labeled with antibodies against CT-1 (left panel) and HIF-1α (right panel) which remained either untreated (a,d) or were treated with menadione (b,e) or H_2O_2 (c,f). (C,D) Embryoid bodies were treated on day 4 of cell culture with either physiological (1% O_2) or chemical ($CoCl_2$) hypoxia and protein (C) and mRNA (D) levels were examined 24 hours later. The images above C show representative embryoid bodies labeled with antibodies against CT-1 (left panel) and HIF-1α (right panel) which remained either untreated (a,d) or were treated with 1% O_2 (b,e) or $CoCl_2$ (50 μM) (c,f). *$P<0.05$, levels are significantly different to those in the untreated controls. Bars, 200 μm.
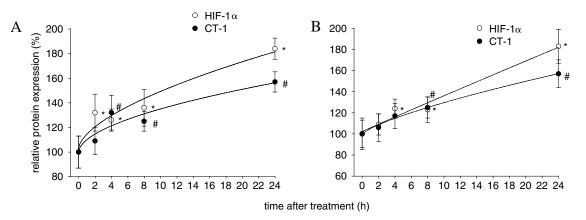
Fig. 4. Time course of HIF-1α and CT-1 upregulation upon treatment with either menadione (A) or CoCl$_2$ (B). Protein expression was assessed 2, 4, 8 and 24 hours after treatment. *,#$P<0.05$, significantly different when compared with levels in untreated samples.

(Fig. 3C,D). Physiological (n=3) as well as chemical hypoxia (n=4) upregulated CT-1 protein expression to 191±18% and 157±13%, respectively, whereas increased levels of HIF-1α protein amounting to 192±20% (n=3) and 183±16% (n=3) were observed upon physiological and chemical hypoxia, respectively (see Fig. 3C). A significant increase in CT-1 as well as HIF-1α mRNA expression amounting to 192±8% (n=3) and 279±24% (n=4), respectively, was only achieved with

chemical hypoxia. Assessment of the time course of HIF-1α and CT-1 expression upon treatment with either menadione (Fig. 4A) (n=3) or CoCl$_2$ (Fig. 4B) (n=4) revealed that upregulation occurred after only 2 hours of incubation. Statistical significance was achieved at earlier time points for HIF-1α expression compared with CT-1 expression, i.e. after 2 and 4 hours in menadione and CoCl$_2$-treated samples, respectively, suggesting that HIF-1α precedes CT-1 expression.
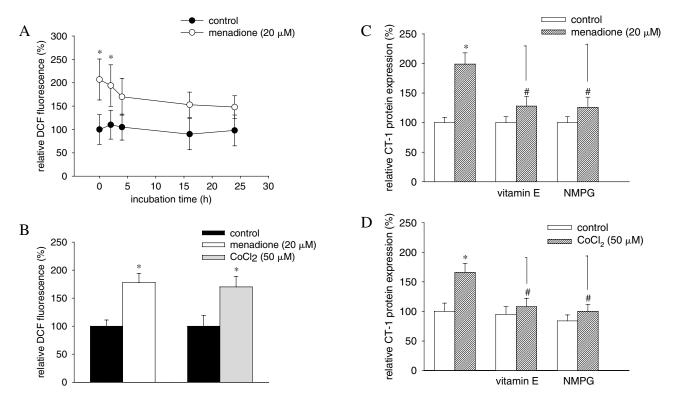

Fig. 5. Generation of ROS following exposure to menadione or chemical hypoxia (CoCl$_2$ treatment). (A) Embryoid bodies were treated for 24 hours with 20 μM menadione. Subsequently, the cell culture medium was exchanged with medium devoid of menadione, and ROS were monitored at different times after removal of menadione. (B) ROS generation in the presence of either menadione (20 μM) or CoCl$_2$ (50 μM). Determination was performed immediately after exposure. The upregulation of CT-1 by menadione (C) and CoCl$_2$ (D) was inhibited by either the free radical scavenger vitamin E (20 μM) or NMPG (20 μM). Embryoid bodies were treated at day 4 of cell culture with either menadione (20 μM) or CoCl$_2$ (50 μM) in the presence of free radical scavengers. After 24 hours CT-1 protein expression was determined by semiquantitative immunohistochemistry. *$P<0.05$, significantly different to levels in the relative controls. #$P<0.05$, significantly different to levels in the CoCl$_2$ or menadione-treated samples before addition of scavengers.

Generation of ROS by menadione and chemical hypoxia in embryoid bodies

Prooxidants as well as chemical hypoxia may increase intracellular ROS generation. When embryoid bodies were treated for 24 hours with 20 µM menadione, a significant increase in ROS generation was observed which remained at an elevated level 24 hours after replacement into normal medium, but was not significantly different from the control after 4 hours (Fig. 5A) (n=4). An increase in ROS generation was likewise achieved when embryoid bodies were treated for 24 hours with CoCl$_2$ (Fig. 5B) (n=3), suggesting that ROS may indeed act as signalling molecules regulating the expression of CT-1 and HIF-1α following treatment with menadione and chemical hypoxia. This was further corroborated by experiments where CT-1 expression following treatment with

menadione (Fig. 5C) (n=3) and CoCl$_2$ (Fig. 5D) (n=4) was assessed in the presence of the free radical scavengers vitamin E (20 µM) and NMPG (20 µM) which resulted in significant downregulation of CT-1 expression, thus indicating regulation of CT-1 expression by intracellular ROS.

Upregulation of NADPH oxidase in embryoid bodies by menadione and hypoxia

Prooxidants may regulate the expression of ROS generating NADPH oxidase thereby providing a feed-forward loop of increased ROS generation even in the absence of external prooxidants. To evaluate this idea, we determined expression of NADPH oxidase subunits. Preincubation for 24 hours with either menadione (20 µM) or CoCl$_2$ (50 µM) significantly increased protein expression of p22-phox, p47-phox, p67-
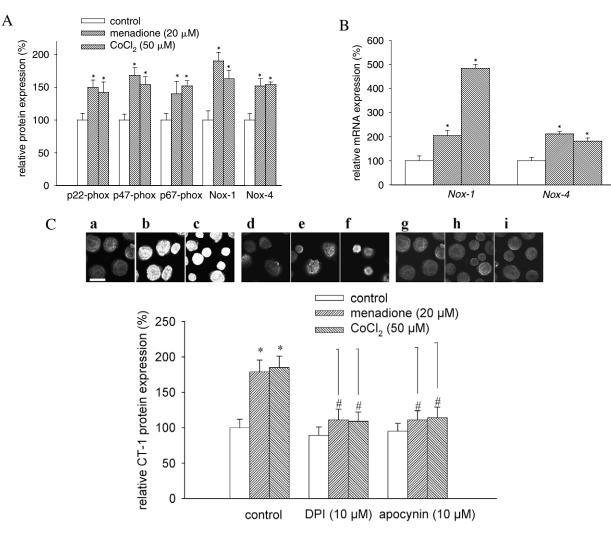

Fig. 6. Upregulation of NADPH oxidase subunits upon incubation of embryoid bodies with menadione and CoCl$_2$. (A) Protein expression of the NADPH oxidase subunits p22-phox, p47-phox, p69-phox, Nox-1 and Nox-4 which was determined 24 hours after treatment of 4-day-old embryoid bodies by semiquantitative immunohistochemistry. (B) mRNA expression of *Nox1* and *Nox4* upon either menadione (20 µM) or CoCl$_2$ (50 µM) treatment. (C) The upregulation of CT-1 by menadione and CoCl$_2$ was inhibited by preincubation with the NADPH oxidase inhibitors DPI (10 µM) or apocynin (10 µM). Embryoid bodies were treated at day 4 of cell culture with either menadione (20 µM) or CoCl$_2$ (50 µM) in the presence of either DPI or apocynin. After 24 hours, CT-1 protein expression was determined by semiquantitative immunohistochemistry. The micrographs are representative embryoid bodies either untreated (a), or treated with menadione (b), CoCl$_2$ (c), DPI (d), DPI + menadione (e), DPI + CoCl$_2$ (f), apocynin (g), apocynin + menadione (h), apocynin + CoCl$_2$ (i). *P<0.05, significantly different from levels in the control. #P<0.05, significantly different to levels in menadione- and CoCl$_2$-treated samples before addition of inhibitors. Bar, 400 µm.

phox, Nox-1 and Nox-4 (Fig. 6A) ($n=4$) as well as mRNA expression of *Nox1* and *Nox4* (Fig. 6B) ($n=5$), suggesting regulation of CT-1 expression by NADPH oxidase-derived ROS. Furthermore, treatment with physiological hypoxia (1% O_2) significantly increased protein expression of p22-phox, p47-phox and Nox-4 (data not shown). The involvement of NADPH oxidase in the regulation of CT-1 expression was further confirmed by incubating embryoid bodies with either menadione (20 µM) or $CoCl_2$ (50 µM) in the presence of the NADPH oxidase inhibitors DPI (10 µM) ($n=3$) or apocynin (10 µM) ($n=3$) (Fig. 6C). This treatment significantly inhibited the increase in CT-1 expression following treatment with either menadione or $CoCl_2$, thus indicating that NADPH oxidase-derived ROS are involved in upregulation of CT-1 by prooxidants and hypoxia.

Expression and phosphorylation of gp130 in embryoid bodies by prooxidants and chemical hypoxia

Regulation of CT-1 expression may be mediated through phosphorylation of the gp130 signal transduction receptor. To assess changes in gp130 expression as well as gp130 phosphorylation in the presence of prooxidants and chemical hypoxia, semiquantitative immunohistochemistry was performed using antibodies against the unphosphorylated as well as the phosphorylated form of gp130. It was found that prooxidants as well as chemical hypoxia significantly increased gp130 expression as well as phosphorylation, suggesting activation of gp130-mediated signal transduction cascades ($n=3$) (Fig. 7A).
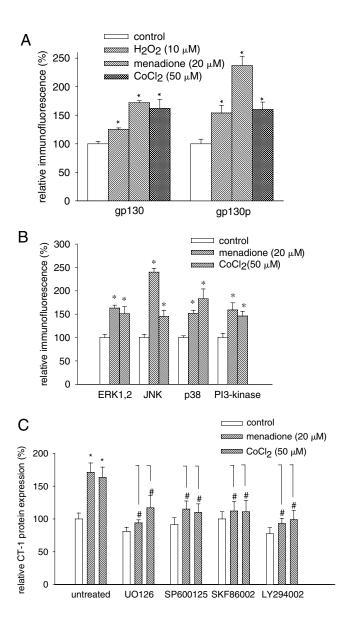
Signalling cascades involved in the upregulation of CT-1 in embryoid bodies by menadione and chemical hypoxia

Upon binding of CT-1 to a LIFR-β:gp130 heterodimer a complex signalling cascade is activated which involves the MAPK members ERK1,2, JNK and p38 as well as PI3-kinase and the Jak/STAT pathway. By using phospho-specific antibodies it was found that phosphorylation of ERK1,2 (menadione, $n=3$; $CoCl_2$, $n=5$), JNK (menadione, $n=3$; $CoCl_2$, $n=5$), p38 (menadione, $n=4$; $CoCl_2$, $n=4$), and PI3-kinase ($n=4$) occurred (Fig. 7B). To correlate the activation of MAPK pathways and PI3-kinase to the expression of CT-1, embryoid bodies were treated with the ERK1,2 inhibitor UO126 (10 µM), the JNK inhibitor SP600125 (10 µM) the p38 inhibitor SKF86002 (10 µM) as well as the PI3-kinase inhibitor LY294002 (20 µM) which significantly downregulated the menadione- and $CoCl_2$-induced upregulation of CT-1 ($n=3$) (Fig. 7C). A comparable inhibition of CT-1 expression was achieved when the Jak/STAT signalling pathway was inhibited with the Jak-2 antagonist AG490 (data not shown).

Absence of cardiomyogenesis and stimulation of CT-1 expression in HIF-1$\alpha^{-/-}$ ES cells

Menadione- and hypoxia-regulated CT-1 expression in ES cells should be absent upon inactivation of HIF-1. To test this notion, embryoid bodies grown from the parental CCE cell line were treated with the HIF-1α inhibitor 2-ME (3 µM). In

Fig. 7. Upregulation of gp130 leads to a complex signalling cascade upon treatment of embryoid bodies with prooxidants or chemical hypoxia. (A) Embryoid bodies were treated at day 4 of cell culture with either H_2O_2 (10 µM), menadione (20 µM) or $CoCl_2$ (50 µM). After 24 hours, gp130 protein expression and phosphorylation was assessed by quantitative immunohistochemistry using an antibody directed against unphosphorylated gp130 or the phosphorylated form of gp130. (B) Activation of ERK1,2, JNK p38 and PI3-kinase upon incubation of embryoid bodies with either menadione (20 µM) or $CoCl_2$ (50 µM). Activation was assessed by semiquantitative immunohistochemistry using phospho-specific antibodies. Maximum activation of ERK1,2 was achieved after 15 minutes; maximum activation of JNK was at 15 minutes for menadione treatment and 30 minutes for $CoCl_2$ treatment; maximum activation of p38 was at 30 minutes for menadione treatment and 60 minutes for $CoCl_2$ treatment; maximum activation of PI3-kinase was at 60 minutes. (C) Inhibition of menadione- and $CoCl_2$-mediated upregulation of CT-1 by the ERK1,2 inhibitor UO126 (10 µM), the JNK inhibitor SP600125 (10 µM), the p38 inhibitor SKF86002 (10 µM), and the PI3-kinase inhibitor LY294002 (20 µM). Embryoid bodies were treated at day 4 of cell culture with either menadione (20 µM) or $CoCl_2$ in the presence of the respective inhibitor. CT-1 protein expression was assessed after 24 hours by semiquantitative immunohistochemistry. *$P<0.05$, significantly different from levels in the untreated control; #$P<0.05$, significantly different to levels before addition of inhibitors as indicated.
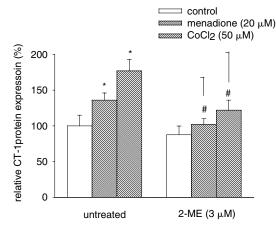
Fig. 8. Upregulation of CT-1 mediated by menadione and CoCl$_2$ is inhibited by the HIF-1α inhibitor 2-ME (3 μM). Embryoid bodies were treated at day 4 of cell culture with either menadione (20 μM) or CoCl$_2$ in the presence of 2-ME. CT-1 protein expression was assessed after 24 hours by semiquantitative immunohistochemistry. *$P<0.05$, significantly different from levels in the untreated control; #$P<0.05$, significantly different to levels before addition of 2-ME.

inhibited the increase in HIF-1α expression observed after menadione (20 μM) exposure (data not shown). To prove the involvement of HIF-1α, we made use of our homozygous HIF-1α-deficient HM ES cell line (Hopfl et al., 2002). In differentiating HIF-1α$^{-/-}$ ES cells cardiomyogenesis was completely absent as assessed by determination of the number of beating embryoid bodies (see Fig. 9A). Moreover, compared with wild-type HM-1 ES cells (see Fig. 9B) and ES cells of the CCE cell line (see Fig. 1A) no upregulation of CT-1 during the time course of differentiation was observed ($n=5$). Furthermore, the upregulation of CT-1 mRNA (see Fig. 9C) ($n=6$) and protein (see Fig. 9D) ($n=4$) following treatment of embryoid bodies with menadione and CoCl$_2$ was completely abolished in HIF-1α$^{-/-}$ ES cells. Taken together, our data strongly support the hypothesis that HIF-1 regulates CT-1 expression.

Discussion

Hypoxia is a (patho)physiological situation occurring during conditions where increased expression of CT-1 has been reported: (1) during cardiac diseases like angina pectoris, cardiac infarction and heart failure (Freed et al., 2003); (2) in the embryonic heart where the heart mass increases through cardiac cell hyperplasia (Wikenheiser et al., 2005); (3) during the growth of ES cells within the three-dimensional tissue of embryoid bodies (Wartenberg et al., 2001). Recently, it has been demonstrated that hypoxia is associated with increased ROS generation produced either through the mitochondrial
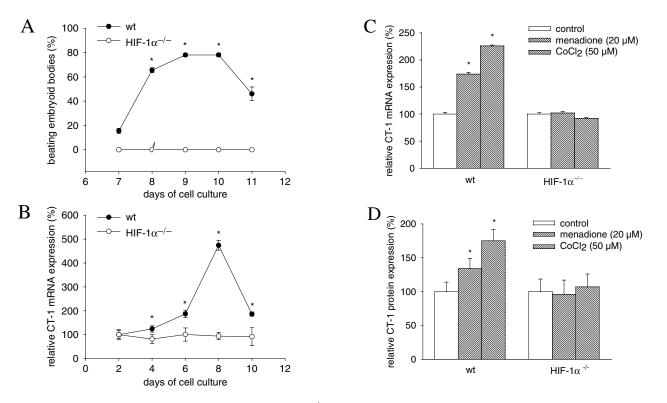
corroboration of our working hypothesis it was apparent that in the presence of 2-ME menadione- and chemical hypoxia-induced upregulation of CT-1 was significantly attenuated (Fig. 8) ($n=5$). Furthermore, treatment with 2-ME significantly

Fig. 9. (A) Cardiac cell differentiation in wild-type (wt) and HIF-1α$^{-/-}$ ES cells. Expression of CT-1 during differentiation (B) and upon incubation with menadione (20 μM) and CoCl$_2$ (50 μM) (C,D). In HIF-1α$^{-/-}$ ES cells cardiomyogenesis was completely absent as evaluated by counting the number of spontaneously contracting embryoid bodies (A). Furthermore, no upregulation of CT-1 mRNA was observed in HIF-1α$^{-/-}$ compared with the wild type (B). Menadione as well as CoCl$_2$ failed to upregulate CT-1 mRNA (C) and protein (D) in HIF-1α$^{-/-}$ cells. *$P<0.05$, significantly different to levels in wild-type embryoid bodies.

respiratory chain or NADPH oxidase activity (Wolin et al., 2005). Elevated ROS are known to occur in cardiac infarction and are suspected to cause ischemia-reperfusion injury (Berg et al., 2005).

Hypoxia has been previously demonstrated to induce CT-1 expression (Hishinuma et al., 1999). Hypoxia occurs in differentiating ES cells within embryoid bodies (Wartenberg et al., 2001) and ROS are generated via NADPH oxidase (Sauer et al., 2000; Sauer et al., 1999), suggesting that hypoxia and ROS may regulate CT-1 expression. Consequently, incubation of ES-cell-derived embryoid bodies with free radical scavengers downregulated CT-1 and HIF-1α expression; by contrast, prooxidants as well as physiological and chemical hypoxia upregulated CT-1 as well as HIF-1α protein and mRNA expression. Furthermore, either menadione treatment or chemical hypoxia resulted in increased ROS generation with subsequent upregulation of ROS-generating NADPH oxidase, supporting the notion that the same ROS-mediated signaling pathways are triggered under conditions of prooxidant incubation and hypoxia.

Elevated ROS levels during hypoxia have been previously shown to occur in pulmonary myocytes (Marshall et al., 1996), cardiac myocytes, Hep3B cells, HeLa cells (Chandel et al., 1998) as well as adipocytes (Carriere et al., 2003); the sources being either the mitochondrial respiratory chain or NADPH oxidase. The data of the present study clearly indicate a distinct role of NADPH oxidase-derived ROS in the regulation of CT-1 expression in ES cells (see Fig. 10) because inhibition of NADPH oxidase by apocyin as well as DPI abolished prooxidant and chemical hypoxia-mediated induction of CT-1. The involvement of ROS in the regulation of CT-1 expression was further validated by experiments demonstrating that the
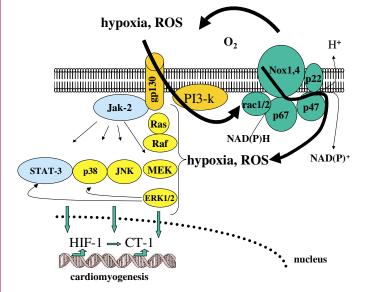
effects observed with the prooxidant menadione and chemical hypoxia were significantly inhibited in the presence of free radical scavengers.

The regulation of cytokine expression by HIF-1α and ROS has been recently discussed (Haddad and Harb, 2005). Previously, stimulation of mRNA expression as well as secretion of IL-6 in response to exposure with prooxidants has been shown (Kosmidou et al., 2002; Kida et al., 2005; Haddad, 2002) and may act in paracrine as well as autocrine manner, thereby activating a loop mechanism disposed to stimulate the IL-6 receptors of the cell. In this respect, the data of the current study demonstrated stimulation of gp130 protein expression as well as phosphorylation by prooxidants and chemical hypoxia, and downstream activation of several members of the previously described (Heinrich et al., 2003), CT-1 activated signal transduction cascade, i.e. the MAPKs ERK1,2, JNK, p38 as well as PI3-kinase, which indeed suggests activation of the CT-1-mediated signaling cascade by ROS (see Fig. 10). Consequently, the data of the present study demonstrated that inhibition of all investigated MAPK pathways, PI3-kinase as well as the Jak/STAT pathway, abolished the prooxidant- and hypoxia-mediated increase in CT-1 and HIF-1α expression.

The mouse *CT-1* gene has been recently isolated. It constitutes 5.4 kilobases (kb) in length and consists of three exons and two introns. When nucleotide sequences of the coding regions of exons were compared with those of the human gene, it was observed that exon 1, 2 and 3 share 96%, 84% and 81% homology, respectively. Interestingly, potential binding sites for several ubiquitous transcription factors including HIF-1 were present in the 5′–flanking region extending 2174 bp upstream from the transcription initiation site (Funamoto et al., 2000).

If CT-1 expression is indeed regulated by HIF-1, absence of hypoxia- and prooxidant-mediated upregulation of CT-1 should be anticipated under conditions of either pharmacological or genetic inactivation of the α subunit of HIF-1. This was investigated by either a pharmacological approach using 2-ME, which has been recently shown to downregulate HIF-1α at the posttranscriptional level and inhibits HIF-1-induced transcriptional activation of VEGF expression (Mabjeesh et al., 2003), or by ES cells homozygous deficient for HIF-1α (Hopfl et al., 2002). Although basal mRNA and protein expression of CT-1 was found in HIF-1$\alpha^{-/-}$ cells (data not shown), upregulation during the time course of ES cell differentiation as occurs in wild-type cells was not observed, and cardiomyogenesis was completely absent. Furthermore, prooxidants as well as chemical hypoxia failed to upregulate CT-1 in HIF-1$\alpha^{-/-}$ ES cells which clearly demonstrates regulation of CT-1 expression by HIF-1.

Although the classical view of HIF-1 regulation proposed stabilization of HIF-1α under hypoxic conditions and downregulation at normoxia by the pVHL-mediated ubiquitin-proteasome pathway, mechanisms of HIF-1α stabilization under normoxic conditions have been recently proposed (Lee et al., 2004). These non-hypoxic pathways are used by many growth factors and cytokines, including insulin-like growth factors (Feldser et al., 1999), transforming growth factor and platelet-derived growth factor (Gorlach et al., 2001) and IL-1β (Hellwig-Burgel et al., 1999), which are all known to utilize ROS as signaling molecules within their signal transduction cascade. In this regard it was demonstrated that inhibition of

Fig. 10. Scheme of the proposed signal transduction cascade activated by ROS and hypoxia. Either hypoxia (initiating elevated ROS generation) or exogenous addition of ROS induces phosphorylation of gp130 and initiates a feed-forward cycle of NADPH oxidase activity and expression. ROS generated by NADPH oxidase act as signalling molecules within the CT-1/gp130 signalling cascade which finally results in HIF-1 upregulation and stimulation of CT-1 expression.

ROS generation abolished hormone and growth-factor-mediated increases in HIF-1α (Richard et al., 2000). The data of the present study demonstrate ROS generation under conditions of either chemical or physiological hypoxia which was recently also reported to occur in intrapulmonary arteries of mice (Liu et al., 2005), in skeletal muscle (Zuo and Clanton, 2005) and in human hepatoma cells (Chandel et al., 2000). This was underscored by the observation that oxygen sensing during hypoxia is dependent on mitochondrial-generated ROS (Brunelle et al., 2005; Emerling et al., 2005).

Regulation of CT-1 expression by HIF-1α and ROS sounds reasonable in light of its biological function in inhibiting cardiac cell apoptosis, promoting cardiac cell hypertrophy and stimulating embryonic cardiac cell differentiation and proliferation which are phenomena occurring under conditions of hypoxia and/or ROS-mediated inflammation. Investigation of the physiological microenvironment that regulates CT-1 expression will not only help us to uncover the role of CT-1 in cardiac hypertrophy and cardiac repair in the infarcted heart, but will also help us to understand the mechanisms of cardiac cell hyperplasia in the embryonic heart and cardiomyogenic differentiation of ES cells.

Materials and Methods

Materials
N-(2-mercapto-propionyl)-glycine NMPG, 2-methoxyestradiol (2-ME), diphenylene iodonium chloride (DPI) and apocynin were purchased from Sigma, Deisenhofen, Germany. Trolox (water-soluble vitamin E), UO126, SP600125, SKF86002, LY294002 and AG490 were from Calbiochem (Bad Soden, Germany).

Spinner-culture technique for cultivation of embryoid bodies
To obtain embryoid bodies, ES cell lines CCE, HM-1 wild type (wt) and HM-1 homozygous deficient for HIF-1α (HIF-1α$^{-/-}$) (Hopfl et al., 2002) were grown on mitotically inactivated feeder layers of primary murine embryonic fibroblasts for a maximum of eight passages in Iscove's medium (Gibco, Life Technologies, Helgerman Court, MD) supplemented with 18% heat-inactivated (56°C, 30 minutes) fetal calf serum (FCS) (Sigma), 2 mM Glutamax, (PAA, Cölbe, Germany), 100 μM β-mercaptoethanol (Sigma, Deisenhofen, Germany), 1% (v/v) NEA non-essential amino acids stock solution (100×) (Biochrom), 0.8% (v/v) MEM amino acids (50×) (Biochrom), 1 mM sodium pyruvate (Biochrom), 0.25% (v/v) penicillin/streptomycin (200×) (Biochrom) and 1000 U/ml LIF (Chemicon, Hampshire, UK) in a humidified environment containing 5% CO_2 at 37°C, and passaged every 2-3 days. At day 0 of differentiation adherent cells were enzymatically dissociated using 0.2% trypsin and 0.05% EDTA in phosphate-buffered saline (PBS) (Gibco), and seeded at a density of 1×10^7 cells/ml in 250 ml siliconized spinner flasks (Integra Biosciences, Fernwald, Germany) containing 100 ml Iscove's medium supplemented with the same additives as described above. After 24 hours, 150 ml medium was added to give a final volume of 250 ml. The spinner flask medium was stirred at 20 rpm using a stirrer system (Integra Biosciences) and 150 ml cell culture medium was exchanged every day.

Immunohistochemistry
Immunohistochemistry was performed with whole-mount embryoid bodies. As primary antibodies a rat anti-mouse CT-1 monoclonal antibody (1:100) (R&D Systems, Wiesbaden, Germany), a mouse anti-mouse HIF-1α monoclonal antibody (1:100) (R&D Systems), a goat anti-mouse gp130 antibody (1:100) (R&D Systems), a phosphospecific polyclonal goat anti-mouse p-gp130 antibody (1:100) (Santa Cruz Biotechnologies, Santa Cruz, CA), polyclonal goat anti-mouse Mox-1 (Nox-1), Nox-4, p22-phox, p47-phox, and rabbit anti-mouse p67-phox antibodies (1:50) (all from Santa Cruz), rabbit polyclonal anti-ERK1,2, anti-JNK, anti-p38 and PI3-kinase P85 antibodies (1:50) directed against the active (phosphorylated) form of the proteins (all from New England Biolabs, Frankfurt, Germany), were used. For HIF-1α, CT-1, gp130, p-gp130, Mox-1, Nox-4, p22-phox and p-67-phox staining the respective tissues were fixed in ice-cold methanol for 30 minutes at –20°C, and washed with PBS containing 0.1% Triton X-100 (PBST) (Sigma). For PI3-kinase P85, p-p38, p-ERK1,2 and p-JNK staining the tissues were fixed for 1 hour at 4°C in 4% formaldehyde in PBS. Blocking against nonspecific binding was performed for 60 minutes with 10% FCS dissolved in 0.01% PBST. The tissues were subsequently incubated overnight at 4°C with primary antibodies dissolved in PBS supplemented with 10% FCS in 0.1% PBST. The tissues were then washed three times with PBST (0.01% Triton) and reincubated with either a Cy5-conjugated goat

anti-rat IgG (H+L), a Cy5-conjugated goat anti-mouse IgG (H+L), or a Cy5-conjugated goat anti-rabbit IgG (all from Dianova, Hamburg, Germany) at a concentration of 3.8 μg/ml in PBS containing 10% FCS in 0.01% PBST. After washing three times in PBST (0.01% Triton) the tissues were stored in PBS until inspection. Fluorescence was recorded using a confocal laser-scanning setup (LSM510 Meta, Carl Zeiss, Germany) connected to an inverted microscope (Axiovert 200M, Carl Zeiss). The confocal setup was equipped with a 5 mW helium/neon laser, single excitation 633 nm (excitation of Cy5). Emission was recorded at >665 nm. The pinhole settings of the confocal setup were adjusted to give a full-width half maximum (FWHM) of 10 μm. Fluorescence was recorded in a depth of 80-120 μm in the depth of the tissue and the fluorescence values in the respective optical section were evaluated by the image analysis software of the confocal setup.

Quantitative RT-PCR
Total RNA from CCE and HM-1 embryoid bodies treated for 24 hours with the substances as indicated was prepared using Trizol (Invitrogen) followed by genomic DNA digestion using DNAse I (Invitrogen, Karlsruhe, Germany). Total RNA concentration was determined by measuring OD_{260nm}. cDNA synthesis was performed using 2 μg RNA in a total volume of 20 μl with MMLV RT (Invitrogen). Primer concentration for qPCR was 10 pM/20 μl. Primer (Invitrogen) sequences were as follows: HIF-1α, 5′-TCA CCA GAC AGA GCA GGA AA-3′ and 5′-CTT GAA AAA GGG GAG AGC CAT CA-3′; CT-1, 5′-GAA CAC ACA GGA CAC AGA TGG AG-3′ and 5′-GAA CAC ACA GGA CAC AGA TGG AG-3′; NOX1, 5′-AAT GCC CAG GAT CGA GGT-3′ and 5′-GAT GGA AGC AAA GGG AGT GA-3′; NOX4, 5′-GAT CAC AGA TCC CTA GCA G-3′ and 5′-GTT GAG GGC ATT CAC CAA GT-3′. Primer sequences for house keeping genes were: BACT, 5′-GAT GAC CCA GAT CAT GTT TGA G-3′ and 5′-CCA TCA CAA TGC CTG TGG TA-3′; GAPDH, 5′-TCG TCC GGT AGA CAA AAT GG-3′ and 5′-GAG GTC AAT GAA GGG GTC GT-3′.

Amplifications were performed in an Icycler Optical Module (Biorad, Munich, Germany) using iQ^{TM} SYBR Green Supermix (Biorad) at 95°C for 15 minutes, 45 cycles of 95°C for 30 seconds and the specific annealing temperatures for 30 seconds then 72°C for 30 seconds and a final extension at 50°C for 10 minutes. Annealing temperatures were 60°C for HIF-1α, Nox-1 and BACT, and 64°C for CT-1, Nox-4 and GAPDH. HIF-1α and Nox-1 data were normalized against the β-actin (BACT) signal, and CT-1 and Nox-4 data were normalized against the GAPDH signal. The primer concentration was 10 pM/20 μl. Fluorescence increase of SYBR Green was automatically measured after each extension step. Amplified transcripts were loaded on a 2% agarose gel. C_T values were automatically obtained. Relative expression values were obtained by normalizing C_T values of the tested genes in comparison with C_T values of the housekeeping genes using the $\Delta\Delta\ C_T$ method.

Measurement of ROS generation
Intracellular ROS levels were measured using the fluorescent dye 2′7′-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) (Molecular Probes, Eugene, OR), which is a nonpolar compound that is converted into a nonfluorescent polar derivative (H$_2$DCF) by cellular esterases after incorporation into cells. H$_2$DCF is membrane impermeable and is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS. For the experiments, embryoid bodies were incubated in serum-free medium and 20 μM H$_2$DCF-DA dissolved in dimethyl sulfoxide (DMSO) was added. After 20 minutes, intracellular DCF fluorescence (corrected for background fluorescence) was evaluated in 3600 μm^2 regions of interest using an overlay mask unless otherwise indicated. For fluorescence excitation, the 488 nm band of the argon ion laser of the confocal setup was used. Emission was recorded using a longpass LP515 nm filter set.

Statistical analysis
Data are given as mean values ± s.d., with n denoting the number of experiments unless otherwise indicated. In each experiment 15-20 embryoid bodies were analyzed. The Student's t-test for unpaired data was applied as appropriate. A value of $P<0.05$ was considered significant.

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