Novel Mechanisms and Functions for Hedgehog Signaling in Development and Disease

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SUMMARY
The developmentally important Hedgehog (Hh) pathway is activated in ischemic tissue and exogenously administered Sonic hedgehog (Shh) supports tissue repair following cardiac ischemia. Hence, it is currently assumed that the endogenous increase in Shh during ischemia serves a beneficial role in limiting cardiac tissue damage. To prove or refute this hypothesis, we treated mice with the Smoothened (Smo) inhibitor cyclopamine to block the Hh pathway during myocardial ischemia-reperfusion. The experimental induction of myocardial ischemia resulted in activation of the Hh pathway, and hallmark features of myocardial damage such as left ventricular dilatation and reduced cardiac output. Unexpectedly, cyclopamine treatment ameliorated left ventricular dilatation and cardiac output. As the beneficial effect of exogenous Shh was suggested to depend on reduced apoptosis, increased vascularization and reduced fibrosis, we subsequently assessed the effect of cyclopamine on these processes. Vascularization was similar in cyclopamine- and control treated animals, but increased apoptosis and reduced fibrosis were observed in the cyclopamine treated animals. Thus, Hh seems to exert a dualistic action in cardiac ischemia in which high exogenous levels are able to foster tissue repair, whereas endogenous Hh seems to be deleterious.

INTRODUCTION
The developmental Hedgehog (Hh) protein family (Nusslein-Volhard and Wieschaus, 1980) is known to be pivotal in many embryonic patterning events and the number of processes in which Hh plays an essential role is expanding persistently. For instance, Indian hedgehog (Ihh) and the more thoroughly characterized homolog Sonic hedgehog (Shh) are critical to the development of the vasculature and of early erythrocytes (reviewed in (Bijlsma et al., 2006a; Byrd and Grabel, 2004)). By virtue of its role in establishing left-right asymmetry, Shh is furthermore crucial to the looping of the cardiac tube, which later becomes the heart.

Recently, it has become clear that the Hh pathway is not only active in the developing embryo, but also in the adult organism (van den Brink et al., 2004). Among others, Hh has been suggested to salvage ischemia-induced tissue damage. Two recent papers of Pola et al. established a strong role for Hh signaling in adult cardiovascular pathophysiology. Shh mediated a profound upregulation of Hh target genes like vascular endothelial growth factor (VEGF) and the angiopoietins Ang-1 and Ang-2, which were found to induce neovascularization (Pola et al., 2001). The authors extended their findings and managed to salvage ischemic hind limbs in mice by injecting Shh in the afflicted muscle (Pola et al., 2003). In another experimental setup, Kusano and colleagues demonstrated that intramyocardial gene transfer of Shh promoted recovery and preservation of left ventricular function in both acute and chronic myocardial ischemia models (Kusano et al., 2005). Hh exerted its beneficial effect by enhancing neovascularization, recruiting bone marrow-derived progenitor cells and reducing fibrosis and cardiac apoptosis.
Interestingly, a characteristic feature of both the hind limb as well as the myocardial ischemia model mentioned above is the endogenous production of Shh and the activation of the associated signaling system. The physiological importance of this endogenously expressed Shh is unexplored in the myocardial model but it is thought that the endogenous increase of Shh during myocardial ischemia serves a beneficial role in limiting tissue damage as it does in hind limb ischemia models. To prove or refute this hypothesis, we set up experiments in which mice were treated with cyclopamine to block the Hh pathway before subjecting them to a model of myocardial ischemia-reperfusion (De Celle et al., 2004). Cyclopamine is an alkaloid that specifically inhibits the activating receptor of the Hh pathway, Smoothened (Smo) (Chen et al., 2002; Incardona et al., 2000; van den Heuvel and Ingham, 1996). In the used model, the left anterior descending coronary artery (LAD) is ligated for 30 minutes and then reperfused for 1 week. End points of this model are dimensional and functional markers for the integrity of the ventricular structure and function. In addition to these physiological parameters, immunohistochemistry was performed to determine the effect of cyclopamine on apoptosis, vascularization, and fibrosis in the heart.

Our results show that in contrast to the prevailing notion that endogenous Shh is a beneficial response following cardiac ischemia, endogenous Shh contributes to ischemia-reperfusion induced injury. Hence, a dualistic role of Shh signaling in coronary disease is revealed, in which high levels of exogenous Shh as well as inhibition of endogenous production are associated with improved outcome of disease.

**RESULTS**

**THE HH PATHWAY IS ACTIVATED IN ISCHEMIC MYOCARDIUM**

Previous data suggest that the Hh pathway is activated in the heart of animals exposed to myocardial ischemia (Kusano et al., 2005). To confirm these observations, we analyzed Patched1 (Ptc1) expression in the heart of mice subjected to myocardial ischemia-reperfusion. Ptc1, the inhibitory regulator of the Hh pathway, is a direct target gene of the Hh pathway and Ptc1 levels are thus indicative of Hh pathway activity (Marigo et al., 1996). As shown in Figure 1A, some baseline expression of Ptc1 was observed in the left ventricle of control animals. Ischemia-reperfusion, however, strongly induced the expression of Ptc1 confirming that indeed the Hh pathway is activated.

**EXCLUSION OF NON-CYCLOPAMINE RESPONSIVE ANIMALS**

Cyclopamine inhibition of Hh signaling is notoriously variable in its efficacy, mainly because of its hydrophobicity (Keeler and Baker, 1989). To eliminate superfluous variation in the experimental setup caused by inefficient cyclopamine bioavailability, we analyzed Hh pathway activity in kidney homogenates of both cyclopamine and solvent control treated animals. Animals in which cyclopamine treatment was unsuccessful will not show decreased Hh pathway activity in organs with an active Hh pathway. As shown in Figure 1B, solvent treated animals (lane 1) show robust expression of Ptc1 (indicative of Hh pathway activity as Ptc1 is a direct target of the Hh pathway). Efficient cyclopamine treatment dramatically reduced Ptc1 expression levels (lane 2), whereas inefficient cyclopamine treatment did not reduce Ptc1 levels (lane 3). Cyclopamine treated animals with Ptc1/β-actin levels (as determined by densitometry) at or above the average Ptc1/β-actin level of all animals were considered non-responders and were excluded from the study (6 out of 16 cyclopamine treated animals).

**FIGURE 1. HH PATHWAY ACTIVITY IN THE ISCHEMIC HEART AND INHIBITION BY CYCLOPAMINE**

(A) Hh pathway activity in sections of control and ischemic myocardium was analyzed by staining for the Hh pathway receptor Ptc1 (staining denoted by arrows). Increased Ptc1 levels, indicating elevated Hh pathway activity, could be seen in left ventricular tissue after 24h reperfusion following 1h ischemia. Indicated is original magnification.

(B) Kidneys of control- and cyclopamine treated animals were homogenized and immunoblotted for Ptc1 and β-actin. Densitometry was performed for all samples and cyclopamine treated animals that did not show diminished Ptc1 levels were considered "non-responder".

82
FIGURE 2. ECHOCARDIOGRAPHY PRE- AND POSTOPERATIVELY

(A) Preoperative functional parameters of the left ventricle as determined by echocardiography are similar between solvent-control and cyclopamine treated animals. Abbreviations: LVSs, systolic left ventricle wall thickness (cm); LVSd, diastolic left ventricle wall thickness (cm); LVIDs, systolic left ventricle internal diameter (cm); LVIDd, diastolic left ventricle internal diameter (cm); FS, fractional shortening (%); SV, stroke volume (μl x 1000); EF, ejection fraction.

(B) Postoperative functional parameters of the left ventricle show improved outcome for cyclopamine-treated animals. (*P<0.05)

(C) Relative values calculated from data in A and B (postoperative value divided by preoperative value) confirm the beneficial effect of cyclopamine (**P<0.01).

(D) Examples of hematoxylin/eosin stained sections confirm left ventricular dilatation in control treated animals compared to non-ischemic animals, which is strongly ameliorated in cyclopamine treated animals.
Echocardiography

To assess the effect of cyclopamine treatment on the function of the afflicted tissue, left ventricular dimensions and resultant functional parameters were obtained by echocardiography. The raw data are shown in Figure 2A and -B (pre- and postoperative, respectively). As evident from Figure 2A, prior to the induction of ischemia-reperfusion, left ventricular integrity and consequent function are similar between cyclopamine and solvent control treated animals. These parameters are similar to those observed after 2 weeks in sham operated animals (De Celle et al., 2004). The induction of ischemia-reperfusion, however, severely compromised left ventricular integrity as evident from both a systolic and diastolic decrease in left ventricle wall thickness (LVS) and an increase of the internal left ventricular diameter (LVID) (Fig. 2B). In addition, ischemia-reperfusion severely reduced fractional shortening, the stroke volume and the ejection fraction, indicating diminished ventricle contractility. The strong deterioration of these parameters highlights the severe effect of the 30 min ischemia-reperfusion procedure on cardiac function.

Figure 2C shows the relative values for the abovementioned parameters as calculated by dividing postoperative values by preoperative values. Cyclopamine treatment reduces left ventricular dilatation as evident from increased systolic and diastolic left ventricle wall thickness (non-significant) as well as from a reduced internal diameter. Furthermore, cyclopamine treatment mitigated the reduction of fractional shortening, stroke volume and ejection fraction induced by ischemia-reperfusion, resulting in postoperative values more similar to preoperative values and thus sham-operated values. Taken
together, the echocardiography data show that cyclopamine treatment significantly restored ventricular function inflicted by ischemia-reperfusion.

To assess the consequence of myocardial ischemia-reperfusion on the histological level, slides were stained by hematoxylin/eosin. As shown in Figure 2D, the left ventricle of mice subjected to ischemia-reperfusion is rather severely dilated. In cyclopamine treated animals, however, the left ventricle seems (almost) unaffected, closely resembling a non-ischemic control ventricle.

Apoptosis

The previously described beneficial effect of exogenous Shh was partly attributed to reduced apoptosis in the infarcted area (Kusano et al., 2005). To determine the effect of endogenous Hh on apoptosis, caspase-3 activity in the left ventricle wall was assessed by immunohistochemistry. As shown in Figure 3, some caspase-3 staining was observed throughout the left ventricle of non-ischemic control animals, whereas ischemia-reperfusion slightly but significantly increased the amount of caspase-3 staining. In the cyclopamine treated animals, caspase-3 staining and thus apoptosis was strongly augmented compared to both non-ischemic controls and solvent treated animals (Fig. 3A and -B). Thus, similar to exogenous Shh, endogenous Hh limits ischemia-reperfusion-induced apoptosis.

To confirm and consolidate the data obtained from the caspase-3 staining, we also employed TUNEL analysis as shown in Figure 3C and D. Hardly any TUNEL-positive nuclei were present in non-ischemic control conditions, whereas the induction of ischemia-reperfusion induced the number of TUNEL positive nuclei (ischemia-control). Cyclopamine treatment further increased the number of TUNEL positive nuclei—and thus apoptotic cells—compared to solvent control treated mice.

Revascularization

Another feature thought to be at least partly responsible for the salvaging effect of exogenously Shh in myocardial ischemia is increased neovascularization of the myocardium. To assess the role of endogenous Shh in vascularization after ischemia-reperfusion injury, heart sections were immunohistochemically stained for α-smooth muscle actin (αSMA, Figure 4A) and positively stained vessels were counted. Solvent treated animals showed αSMA positive vessels throughout the heart. Cyclopamine treatment did not reduce the number of
apoptosis (Thayer et al., 2003). On the contrary, apoptosis inhibited by Shh is directly dependent on Ptch1, which serves as a dependence receptor, and does not necessarily involve downstream pathway signaling (Thibert et al., 2003).

The beneficial effect of cyclopamine on myocardial injury as assessed by echocardiography was unambiguous, but we were unable to conclusively identify the underlying mechanism responsible. Cyclopamine did not significantly affect vascularization. Considering the relatively short time frame of the experiment and the fact that neovascularization is thought to be more important in chronic models of myocardial ischemia, our observation might not be that surprising (Kusano et al., 2005).

In analogy, the presented study has some limitations that should be kept in mind when interpreting our data. For instance, the model we use does not allow us to discriminate between the effects of cyclopamine on acute myocardial injury versus the effects on the subsequent remodeling. Future studies in which the effect of cyclopamine on ischemia-reperfusion-induced myocardial damage is assessed at different time-points should pinpoint whether cyclopamine exerts its beneficial action in the acute or remodeling phase. Furthermore, we did not determine the area at risk (i.e. ischemic area) in the individual animals. Formally, it is therefore difficult to exclude that cyclopamine-treated mice accidentally had smaller infarctions and thereby exhibited better postischemic functional recovery. However, the LAD ligation was performed by a single experienced technician in a blinded fashion minimizing variation in area at risk (and infarct size) and we feel confident that infarct sizes were similar between groups.

Interestingly, the fibrosis data might shed another light on the potential beneficial effect of apoptosis induction by cyclopamine (as discussed above). One could argue that the induction of apoptosis keeps fibroblast proliferation in check, thereby limiting ischemia-reperfusion-induced fibrosis and consequently myocardial damage. Future experiments should test the validity of this hypothesis.

Whatever the mechanism responsible for the protective effect of cyclopamine, it is fascinating to speculate about the clinical implications of this finding. Systemic administration of a Hh-inhibitory molecule might be a more practical treatment option than the previously utilized injection of Shh expressing plasmid directly into the myocardium. As systemic administration of cyclopamine has so far not been successful in humans, (pro-)vitamin D3, which is well-tolerated at high doses, might be an attractive therapeutic option (Bijlsma et
al., 2006b). However, before making such claims, studies addressing the actual potential of (pro-)vitamin D in experimental myocardial ischemia need to be performed. Currently, we are planning these experiments in addition to experiments in which Hh inhibitors are administered after induction of ischemia, which should more closely mimic the clinical situation.

In conclusion, we show that cyclopamine treatment limits myocardial ischemia-reperfusion injury. Thus, Hh seems to exert a dualistic action in cardiac ischemia in which high exogenous levels are able to foster tissue repair, whereas endogenous Hh seems to aggravate ischemic disease.

MATERIALS AND METHODS

ANIMALS

Ten week old, male C57Bl/6 mice (Harlan Sprague-Dawley Inc., The Netherlands), weighing approximately 25g at the time of surgery, were maintained at the animal care facility of the University of Maastricht according to institutional guidelines, with free access to food and water. Animal procedures were carried out in compliance with Institutional Standards for Humane Care and Use of Laboratory Animals. The Animal Care and Use Committee of the Maastricht University approved all experiments.

EXPERIMENTAL SET-UP

Mice were anaesthetized with ketamine (100 mg/kg im) and xylazine (5 mg/kg sc). Body temperature, monitored with a rectal probe, was maintained at 37°C using a warming pad and heating lamp. The trachea of each mouse was intubated per orally with a stainless-steel tube connected to a respirator (Hugo Sachs Electronic, March-Hugstetten, Germany) set at a stroke volume of 250 μl and a rate of 210 strokes/min. After left thoracotomy and exposure of the heart, the left anterior descending coronary artery (LAD) was ligated with 6–0 (metric 0) polypropylene suture (Surgipro, Chicago, IL) just proximal to its main branching point. The suture was tied around a 3 mm long polyethylene tube (PE-10) to induce ischemia. Under microscopic viewing blood flow was re-established after 30 min of ischemia by removal of the tube. For Figure 1A, ischemia was induced for 1h, and reperfusion was allowed for 24h.

Animals were injected intraperitoneally with 2 mg/kg cyclopamine (a dose previously shown to completely block the Hh pathway (van den Brink et al., 2001; Biomol, Plymouth Meeting, PA) or solvent control (2-hydroxypropyl-β-cyclodextrin) 1 day before ligation of the coronary artery. Immediately before the surgical procedure, echocardiography was performed to determine left ventricular dimension and function (pre-MIR). Following the operation, animals were treated daily for 7 days with cyclopamine (or solvent) after which a second echocardiography was performed prior to sacrificing the animals (post-MIR). Upon sacrifice, hearts were removed for (immuno)histochemistry whereas kidneys were collected for assessing Hh pathway activity to determine the efficacy of cyclopamine treatment. The technicians performing the (randomized) treatment, operation and analysis were blinded for the identity of the injected solutions.

(Immuno)Histochemistry

Hearts were fixed in 10% buffered formalin and embedded in paraffin. Slides (4 μm thick sections) were stained with hematoxylin/eosin according to routine procedures or incubated in 1% Sirius Red F3B in picric acid for 1h to assess fibrosis. For immunohistochemistry, slides were first deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 0.03% H2O2, in methanol, antigen retrieval was performed by boiling for 5 minutes in 10 mM citrate buffer (pH 6.0). Slides were blocked with TENG-T. Ptc1 staining was performed with 1:250 α–Ptc1 G-19 (Santa Cruz Biotechnology, Santa Cruz, CA). Specific active caspase-3 staining was performed with 1:200 α–ACTIVE Caspase-3 antibody (Promega, Madison, WI). TUNEL staining was performed according to manufacturer’s protocol (DeadEnd Colorimetric TUNEL System, Promega). Smooth muscle actin was visualized using anti-α-SMA-1 clone 1A4 (1:200, Sigma-Aldrich, St Louis, MO). Slides were incubated with the appropriate HRP-conjugated secondary antibodies and DAB staining was used to visualize peroxidase activity. Slides were photographed on a Zeiss Axioskop (Zeiss, Jena, Germany) with a Sony DXC950P CCD camera (Sony, Tokyo, Japan). Caspase staining was quantified by measuring staining intensity; image colors were inverted in Photoshop 7.0 (Adobe Systems, San Jose, CA) and luminosity was determined in 3 separate areas in the left ventricle. For TUNEL staining, positive nuclei were counted per optical field at random locations in the left ventricle wall. For quantification of vascularization anti-a-SMA-1 stained vessels were counted in the heart. For assessment of amount fibrosis in the ventricle wall, the fraction of Sirius Red-stained ventricle wall thickness was measured at 3 sites in the ventricle wall.

Western Blot

Kidneys were collected and homogenized in Greenberger lysis buffer. Protein content was determined using a BCA protein assay (Pierce Biotechnology, Rockford, IL), equalized, and samples were diluted with 2x Laemmli buffer. After heating, samples were run on 10% SDS-PAGE gels, and subsequently transferred to blotting membrane. Following blocking with 5% Protiﬁ (Nutricia, Zoetermeer, the Netherlands) in TBS-T, membranes were incubated overnight in 1:2000 α–β-actin (Santa Cruz Biotechnology) or 1:500 α–Ptc1 (G-19, Santa Cruz Biotechnology). Membranes were incubated in α–rabbit secondary antibody (1:2000, Dako Cytomation, Glostrup, Denmark) and imaged on a LAS3000 dark box (Fujifilm, Tokyo, Japan). Densitometry was performed in Photoshop using the histogram function in a selected area of constant size for each band. Background was subtracted and values for Ptc1 were corrected for those of β-actin.

Echocardiography

Transthoracic echocardiography of the left ventricle (LV) was performed under light isoflurane anesthesia using a Hewlett-Packard 15 MHz linear array transducer (5–6 l) interfaced with a Sonos
5500 echocardiography system (Philips, Eindhoven, the Netherlands). Two-dimensional B-mode echocardiograms were captured at a rate of 90–120 Hz from parasternal long-axis views as well as from midpapillary short-axis views of the left ventricle. Data were obtained from at least three different images taken in end diastole and peak systole using EnConcert software (Agilent Technologies, Andover, MA).

**Statistical analysis**

Depicted are the means ± SEM. After exclusion of non-responders (see result section for details), 7 animals remained for the control groups and 8 animals for the cyclopamine group. Comparison between two groups was done using unpaired student’s t-test.

**References**


