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An Old Dog Learns a New Trick: Netrin-1 in Cardioprotection

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Netrins are a family of proteins that direct cell and axon migration during development [1]. Three secreted netrins (netrin-1, -3 and -4) have been identified in mammals, in addition to the GPI-anchored membrane proteins, netrin-G1 and G2 [1]. Netrin-1, a diffusible protein produced by floor plate cells, was initially identified for its ability to guide axons [2]; however, widespread expression of netrin-1 and its receptors outside the nervous system suggests additional roles for these ligand/receptor pairs during morphogenesis [1,3]. Indeed, netrin-1 and its receptors have been implicated in other developmental processes, including regulation of apoptosis [4], leukocyte migration [5], tumorigenesis [4], vascular remodeling and vessel navigation [3,6]. Despite the tremendous advances in examining the role of netrin-1 in the neuronal development and more recently, vascular development, there remains a substantial need to extend our understanding of netrin-1's effects on cardiovascular function. In their article, Zhang J. and Cai H. discovered that the cardioprotective effects of netrin-1 in myocardial ischemia-reperfusion (I/R) injury are through a DCC/ERK1/2/eNOS_{s1177}/NO/DCC feed-forward mechanism. Thus, only previously defined as a neuronal developmental protein and a regulator of cardiovascular development, a new role of netrin-1 is uncovered as a potent cardioprotective agent.

1. Nitric Oxide in Myocardial I/R

Over two decades of research has shown the involvement of nitric oxide (NO) in cardiovascular biology [7]. Nitric oxide synthase (NOS) generates NO by converting L-arginine to L-citrulline [7]. The majority of animal studies have shown cytoprotective effects of NO against I/R injury. In the absence of endothelial NOS (eNOS), myocardial I/R injury was exacerbated [8]. On the contrary, organic NO donors demonstrated cardioprotection in myocardial I/R [9]. However, a couple of studies have shown the cytotoxic effects of NO [10–11]. An explanation for the dichotomous effects of NO may lie in its complex interaction with reactive oxygen species (ROS), which is particularly pertinent in the context of I/R, where oxidative stress is significantly enhanced [12]. NO can interact with superoxide to form the potent oxidant peroxynitrite, which is toxic to cardiac myocytes [13]. The greater availability of superoxide may therefore favor peroxynitrite production and toxicity. Thus, superoxide may be an important rate limiting factor determining the protective versus toxic effects of NO [14].

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Previously, Cai's laboratory found that netrin-1 stimulated NO production in mature endothelial cells (ECs) in a deleted in colorectal cancer (DCC)-dependent manner [6]. This finding shares similarities with their current observation by Zhang et al. that netrin-1 induced an approximate 2-fold increase in cardiac NO production, which was associated with a 49% decrease in infarct size following I/R injury. The specific NO-mediated cardioprotective effect of netrin-1 was abolished by NO scavenger PTIO and NOS inhibitor L-NAME. Thus, the present study by Zhang et al. establishes an intermediate role of NO in netrin-1 provoked cardioprotection.

2. Source of NO in the Heart

At present, there are three known isoforms of NOS designated: 1) neuronal NOS (nNOS or NOS 1), 2) inducible NOS (iNOS or NOS 2), and 3) endothelial NOS (eNOS or NOS 3) [15]. The mammalian myocardium has been found to express both eNOS and nNOS. eNOS is found in coronary and endocardial endothelial cells and cardiomyocytes [16] whereas nNOS has been localized to cardiac autonomic nerves and ganglia and cardiomyocytes [17–18]. The eNOS and nNOS derived-NO is well known for its paracrine/autocrine effects on cardiomyocytes [19]. Furthermore, the high output iNOS isoform may also express in many different cells (e.g., endothelial cells, cardiomyocytes, and inflammatory cells) in pathological settings [20]. Zhang et al. examined expression of three isoforms of NOS in the heart. iNOS and nNOS levels were unaffected by I/R. However, eNOS expression was reduced in I/R heart and netrin-1 increased eNOS levels. The reduced eNOS expression and phosphorylation are clearly associated with decreased NO levels during I/R. In netrin-1 perfused post-I/R heart, NO level was increased. Furthermore, some of the NO staining was detected specifically in the ECs that line cardiac microvessels. The rest of the NO staining seemed to come primarily from cardiomyocytes, and showed an increase in netrin-1-treated myocardium. Additionally, in primarily cultured cardiomyocytes, netrin-1 treatment also increased NO production, suggesting the crucial role of cardiomyocytes-derived NO in cardioprotection.

Importantly, in recent years, it has been suggested that the regulation of NO synthesis by eNOS in the cardiomyocyte represents a critical pathway to explain the benefits of several effective treatments for both acute myocardial ischemia and chronic congestive heart failure [21–22]. This study further demonstrated that netrin-1-induced cardioprotection is attributed to the activation of cardiomyocyte eNOS in addition to that of endothelial cell eNOS.

3. Netrin-1 Signaling during I/R

To carry its functions, netrin-1 interacts with specific receptors that belong to three protein families: DCC/neogenin, UNC5 and DSCAM. The work by Zhang et al. for the first time examined expression profiles of the eight known netrin-1 receptors: DCC, neogenin, UNC5A-D, integrin $\beta 1$, $\beta 4$, $\alpha 3$, and $\alpha 6$ using heart tissue [1]. Among them, DCC, neogenin, UNC5A, integrin $\beta 1$ and $\alpha 3$ were found highly expressed in C57BL/6J mouse hearts.

Previously, the same Cai's laboratory reported for the first time novel mechanisms whereby netrin-1 potently stimulates angiogenesis in mature ECs, which involve a DCC-dependent eNOS activation [6]. This study provided further support for an essential role of DCC in mediating netrin-1 induction of NO in cardiac ECs and myocytes. Netrin-1 increased ERK1/2 and eNOS_{s1177} phosphorylation, and DCC protein expression, which was diminished by I/R. Netrin-1's protective effects on infarct size and NO production was attenuated by pre-perfusion with DCC-antibody, U0126 (MEK1/2 inhibitor), L-NAME or PTIO (NO scavenger). Thus, netrin-1/DCC/ERK/eNOS signaling account for netrin-1 mediated cardioprotection following I/R. Furthermore, netrin-1-induced DCC upregulation was NO and ERK1/2-dependent, evidenced by reduced DCC production following U0126 or L-NAME treatment, implicating a feed-forward mechanism. The above mechanisms were also confirmed in DCC $\pm$ mice and

isolated cardiomyocytes. Thus, the work by Zhang et al. neatly identifies a netrin-1/DCC/ERK1/2/eNOS_{s1177}/NO/DCC feed-forward loop that mediates the cardioprotective effects of netrin-1.

4. Netrin-1 in Cardiovascular Pathophysiology

Despite having been well characterized for its role in regulating axonal guidance either as attractants or repellents [23–25], the effects netrin-1 on cardiovascular pathophysiology remain to be largely elusive. Recent studies have highlighted the anatomic and structural similarities between blood vessels and nerves [26]. The two networks are often aligned, with nerve fibers and blood vessels following parallel routes. Thus, netrin-1, an axon guidance cue used to guide axon path-finding and regulate neuron proliferation, may also be involved in the angiogenesis respecting the anatomical similarity of neural and vascular system.

Angiogenesis is an important process required for blood vessel formation and plays an important role in cancer [27–28]. Interestingly, netrin-1 also showed a dual role in regulating angiogenesis by either stimulatory [29–31] or inhibitory effects [32]. Cai's laboratory reported that netrin-1 induces angiogenesis via a DCC-dependent ERK1/2-eNOS pathway in mature endothelial cells [6]. On the other hand, Lu et al. [33] have shown that netrin-1 causes endothelial filopodial retraction, and disruption of the UNC5B gene in mice leads to aberrant extension of endothelial tip cell filopodia, excessive vessel branching and abnormal navigation of developing capillaries. Yang et al. [34] suggested that netrin-1 produces either facilitative or inhibitory effects on angiogenesis depending upon its concentration. Moreover, target knockdown of UNC5B in vascular endothelial cells, using a specific siRNA, resulted in a significant increase in cell proliferation and migration along with a loss of the inhibitory effect, regardless of concentration. Thus, netrin-1, along with its receptors, has been found to act as attractants or repellents in both nervous and vascular system.

In addition to the role of netrin-1 in cardiovascular development, recent studies suggested a protective role of netrin-1 in renal I/R injury that is mediated by reductions in leucocytes infiltration, cytokine expression, oxidative stress, and apoptosis, as well as an increase in proliferation [35–36]. The expression of netrin-1 receptor UNC5B, but not DCC, was also increased initially, but decreased later during renal I/R injury. Whether these observations represent NO-regulated downstream mechanisms (note ERK1/2 activation in the heart occurs as rapid as 30 min after netrin-1 infusion, see suppl. Fig. 3E) that are similar to netrin-1 mediated cardioprotection following cardiac I/R injury, remain to be further elucidated.

In summary, this study by Zhang and Cai for the first time demonstrated a cardioprotective role of netrin-1 through DCC/ERK/eNOS_{s1177}/NO/DCC feed-forward signaling in cardiac ECs and myocytes. The study on the cardiovascular effects of netrin-1 is still at its very early stage, but NO-mediated cardioprotection induced by netrin-1 represents new potential targets and suggest novel therapeutic opportunities. Future studies are warranted to examine the effects of netrin-1 on cardiac functional changes in *in vivo* model with either acute or chronic I/R injury. Systemic effects of netrin-1 administration may also need to be examined to evaluate its safety and therapeutic potential in the future.

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