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Calcium channel blockers ameliorate iron overload-associated hepatic fibrosis by altering iron transport and stellate cell apoptosis

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ABSTRACT

Liver fibrosis is the principal cause of morbidity and mortality in patients with iron overload. Calcium channel blockers (CCBs) can antagonize divalent cation entry into renal and myocardial cells and inhibit fibrogenic gene expression. We investigated the potential of CCBs to resolve iron overload-associated hepatic fibrosis. Kunming mice were assigned to nine groups ($n = 8$ per group): control, iron overload, deferoxamine, high and low dose verapamil, high and low dose nimodipine, and high and low dose diltiazem. Iron deposition and hepatic fibrosis were measured in mouse livers. Expression levels of molecules associated with transmembrane iron transport were determined by molecular biology approaches. In vitro HSC-T6 cells were randomized into nine groups (the same groups as the mice). Changes in proliferation, apoptosis, and metalloproteinase expression in cells were detected to assess the anti-fibrotic effects of CCBs during iron overload conditions. We found that CCBs reduced hepatic iron content, intracellular iron deposition, the number of hepatic fibrotic areas, collagen expression levels, and hydroxyproline content. CCBs rescued abnormal expression of α1C protein in L-type voltage-dependent calcium channel (LVDCC) and down-regulated divalent metal transporter-1 (DMT-1) expression in mouse livers. In iron-overloaded HSC-T6 cells, CCBs reduced iron deposition, inhibited proliferation, induced apoptosis, and elevated expression of matrix metalloproteinase-13 (MMP-13) and tissue inhibitor of metalloproteinase-1 (TIMP-1). CCBs are potential therapeutic agents that can be used to address hepatic fibrosis during iron overload. They resolve hepatic fibrosis probably correlated with regulating transmembrane iron transport and inhibiting HSC growth.

1. Introduction

Iron is both essential and toxic (Wosten et al. 2000). Globally, the prevalence of excessive body iron or iron overload has reached epidemic levels, whether caused by primary (hereditary) hemochromatosis or secondary iron overload (e.g., alcoholic liver diseases, chronic viral hepatitis, and thalassemia) (Merryweather-Clarke et al. 1997; Oudit et al. 2003; Deugnier et al. 2008). Iron overload leads to the accumulation of iron into tissues and tends to follow distinct patterns where the liver is a common site of deposition; this leads to diseases that range from elevated aminotransferase levels to hepatic fibrosis, one of the leading causes of morbidity and mortality in patients with primary hemochromatosis (Witzleben and Chaffey 1962; Pietrangelo 2004; Fargion et al. 2011). Although iron chelators, such as deferoxamine (DFO), are available for removing excess iron, various side effects and

Abbreviations: CCB, calcium channel blocker; HSCs, hepatic stellate cells; DFO, deferoxamine; Tf, transferrin; TfR, transmembrane transferrin receptor; Ft, ferritin; LVDCCs, L-type voltage-dependent calcium channels; DMT-1, divalent metal transporter-1; ECM, extracellular matrix; Cont, control; Fe, iron overload; H-Ver, high-dose verapamil; L-Ver, low-dose verapamil; H-Nim, high-dose nimodipine; L-Nim, low-dose nimodipine; H-Dil, high-dose diltiazem; L-Dil, low-dose diltiazem; DMSO, dimethyl sulfoxide; $FeSO_4$, ferrous sulfate; FAAS, flame atomic absorption spectrometry; TEM, transmission electron microscopy; MMP-13, matrix metalloproteinase-13; TIMP-1, tissue inhibitor of metalloproteinase-1.

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cumbersome administration schedules have limited their long-term success (Sheth 2014).

Under normal conditions, human iron homeostasis relies on the harmonious functions of several complicated regulatory systems. Hepcidin, a peptide hormone produced by hepatocytes, is the central regulator of systemic iron homeostasis (Nemeth et al. 2004). Extracellular iron in the ferrous state (Fe^{2+}) circulates in the plasma when coupled with high-affinity transferrin (Tf). Cells can absorb Tf-dependent iron through the transmembrane Tf receptor (TfR) and then store iron in cytoplasm as ferritin (Ft) (Hentze et al. 2010). Although the Tf-dependent pathway of iron uptake predominates under normal circumstances, pathological iron overload leads to supersaturation of Tf, and excess iron can then exist in a free and insoluble form (Fe^{2+}). Tf-independent iron, which is incompatible with either iron delivery in plasma or iron traffic inside cells, can then deposit locally in the tissue and injure cellular membranes and organelles (Brissot et al. 2012).

Targeted therapy to control prolonged insults has established that advanced fibrosis can regress (Friedman 2008b). Therefore, reducing free iron uptake into hepatocytes is critical for treating iron overload-associated fibrosis. Studies on the existence of Tf-independent routes of iron uptake suggest that calcium channel blockers (CCBs) play a crucial role in antagonizing free iron (Fe^{2+}) entry into cardiomyocytes (Tsushima et al. 1999a; Oudit et al. 2003; Ludwiczek et al. 2007; Fernandes et al. 2013). Both L-type voltage-dependent calcium channels (LVDCCs) and divalent metal transporter-1 (DMT-1) can transport a number of divalent cations, including Fe^{2+}, Zn^{2+}, Co^{2+}, Sr^{2+}, Ba^{2+}, and Mn^{2+} into cells (Ludwiczek et al. 2007). Our previous study identified the therapeutic potential of CCBs for nephrotoxicity induced by lead, which might share some of the same routes with iron influx (Zhang et al. 2013a). Moreover, CCBs have been applied to clinical treatments for myocardiopathy patients with iron overload and may enhance the efficacy of heart iron removal (Fernandes et al. 2013).

Hepatic stellate cells (HSCs) have been recognized as the major extracellular matrix (ECM)-producing cell type in the liver (Kharbanda et al. 2004), and their activation correlates well with the up-regulation of their LVDCCs; activated HSCs possess a large number of LVDCCs (Bataller et al. 1998; Roth-Eichhorn et al. 1999; Bataller et al. 2001). In experimental studies, CCBs have been shown to exert antifibrotic effects (Bataller et al. 1998; Roth-Eichhorn et al. 1999; Bataller et al. 2001; Matsui et al. 2010; Boggio et al. 2011; Matsuda et al. 2011; Shafik et al. 2011). Our previous studies demonstrated that Salvia miltiorrhiza (a traditional Chinese herbal medicine) is effective in decreasing iron deposition and inhibiting fibrotic development in iron-overloaded mice (Gao et al. 2013; Zhang et al. 2013b), which is likely attributable to its ability to inhibit L-type calcium currents (as a CCB) (Gao et al. 2014). Therefore, CCBs might be novel agents for pharmacological therapy directed against iron-overload associated fibrosis in the liver, because of their antifibrotic and iron antagonist properties.

In this study, based on an iron-overloaded mice model previously established in our laboratory (Gao et al. 2013; Zhang et al. 2013b), we identified the antifibrotic properties of CCBs in iron-overloaded mice and HSC-T6 cells. This work has helped to identify the underlying CCB mechanisms involved in liver iron transport and HSC-T6 cells growth. Our results support the idea that CCBs might be effective for iron-overloaded patients with hepatic fibrosis.

2. Materials and methods

2.1. Chemicals

Verapamil hydrochloride injection was supplied by Harvest Pharmaceutical Industry Co., Ltd. (Shanghai, China). Nimodipine injection was purchased from Fangming Pharmaceutical Industry Co., Ltd. (Shandong, China). Diltiazem hydrochloride injection was provided by Heping Pharmacy Co., Ltd. (Guangzhou, China). Iron dextran injection purchased from Sunaccord Biological Technical Co. Ltd. (Hunan, China). DFO was supplied by Novartis Pharma AG (Basel, Switzerland). All other reagents were purchased from Sigma (Shanghai, China).

2.2. Animals

Healthy male Kunming mice (8 weeks-old; bodyweight, 20.0 ± 2.0 g) were housed under a 12 h light/dark cycle at controlled temperature and humidity with free access to food and water. The study was authorized by the Animal Ethics and Use Committee of the Hebei Science and Technical Bureau in China and was in compliance with National Institute of Health Guide for Care and Use of Laboratory Animals (approval number: HBTCM-2013-11; approval date: May 17, 2013). All animals were bred under pathogen free conditions in the Central Laboratory of Hebei University of Chinese Medicine's animal facilities.

2.3. Experimental protocol

After one week of acclimation, a total of 72 Kunming mice were randomized into nine groups of eight mice each: control (Cont), iron overload (Fe), high-dose verapamil (H-Ver), low-dose verapamil (L-Ver), high-dose nimodipine (H-Nim), low-dose nimodipine (L-Nim), high-dose diltiazem (H-Dil), low-dose diltiazem (L-Dil), and DFO groups. Mice in the non-control groups received an iron dextran injection (Sunaccord, Hunan, China) at 50 $mg \cdot kg^{-1}$ (Zhang et al. 2013b; Oudit et al. 2003), while the control group received isovolumic saline. Mice in the H-Ver, L-Ver, H-Nim, L-Nim, H-Dil, and L-Dil groups received either 4 $mg \cdot kg^{-1}$ (high; H) or 1 $mg \cdot kg^{-1}$ (low; L) of verapamil hydrochloride injection (Harvest, Shanghai, China), nimodipine injection (Dongyao, Shandong, China), or diltiazem hydrochloride injection (Heping, Guangzhou, China) 4 h before the administration of iron, while mice in the Cont and Fe groups received isovolumic saline. Mice in DFO group (positive control) were given desferrioxamine mesilate by injection (Novartis, Basel, Switzerland) at a dose of 100 $mg \cdot kg^{-1}$ 4 h before the administration of iron (Zhang et al. 2013b; Oudit et al. 2003). Treatments were administered by peritoneal injection once every other day for nine weeks. At the end of the experiment, the mice were anesthetized with sodium pentobarbital (50 $mg \cdot kg^{-1}$), and the liver was excised and examined as described below.

2.4. Cell culture and grouping

HSC-T6 cells, an immortalized rat HSCs line (Vogel et al. 2000), exhibit the same phenotypic characteristics as the activated HSCs (Kim et al. 1998). HSC-T6 cells were provided by Prof H.Q. Jiang (The Second Hospital of Hebei Medical University, Shijiazhuang, China). HSC-T6 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) and supplemented with 10% fetal bovine serum (FBS, BI, USA) and 100 $U \cdot ml^{-1}$ penicillin/streptomycin (Gibco, USA), as previously described (Vogel et al. 2000). The cells were also randomized into 9 groups as was done for the mice. The drugs were dissolved in dimethyl sulfoxide (DMSO), diluted, and added to the culture medium. Cells in H-Ver, L-Ver, H-Nim, L-Nim, H-Dil and L-Dil groups were incubated with verapamil, nimodipine or diltiazem at 30 μM (high dose) and at 10 μM (low dose), while cells in last group were given 1 μM DFO, and cells in Cont and Fe groups were given isovolumic DMSO. Four hours later on the same day, cells in the latter eight groups were incubated with ferrous sulfate ($FeSO_4$) at 1 μM and vitamin C at 5 mM for 24 h, while cells in control group were given DMSO. Cell samples were harvested 24 h after the addition of $FeSO_4$. All experiments were performed in triplicate and repeated at least three times.

2.5. Flame atomic absorption spectroscopy (FAAS)

To determine whether CCBs affect iron deposition in the liver, we quantified the tissue iron content by FAAS. To prepare frozen hepatic homogenates for FAAS, the samples were dried at 65 °C, weighed and