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Nicotinamide-rich diet protects the heart against ischaemia–reperfusion in mice: A crucial role for cardiac SUR2A

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ABSTRACT

It is a consensus view that a strategy to increase heart resistance to ischaemia–reperfusion is a warranted. Here, based on our previous study, we have hypothesized that a nicotinamide-rich diet could increase myocardial resistance to ischaemia–reperfusion. Therefore, the purpose of this study was to determine whether nicotinamide-rich diet would increase heart resistance to ischaemia–reperfusion and what is the underlying mechanism. Experiments have been done on mice on control and nicotinamide-rich diet (mice were a week on nicotinamide-rich diet) as well as on transgenic mice overexpressing SUR2A (SUR2A mice), a regulatory subunit of cardioprotective ATP-sensitive K^+ (K_{ATP}) channels and their littermate controls (WT). The levels of mRNA in heart tissue were measured by real-time RT-PCR, whole heart and single cell resistance to ischaemia–reperfusion and severe hypoxia was measured by TTC staining and laser confocal microscopy, respectively. Nicotinamide-rich diet significantly decreased the size of myocardial infarction induced by ischaemia–reperfusion (from $42.5 \pm 4.6\%$ of the area at risk zone in mice on control diet to $26.8 \pm 1.8\%$ in mice on nicotinamide-rich diet, $n = 6$–12, $P = 0.031$). The cardioprotective effect of nicotinamide-rich diet was associated with 11.46 ± 1.22 times ($n = 6$) increased mRNA levels of SUR2A in the heart. HMR1098, a selective inhibitor of the sarcolemmal K_{ATP} channels opening, abolished cardioprotection afforded by nicotinamide-rich diet. Transgenic mice with a sole increase in SUR2A expression had also increased cardiac resistance to ischaemia–reperfusion. We conclude that nicotinamide-rich diet up-regulate SUR2A and increases heart resistance to ischaemia–reperfusion.

1. Introduction

Sarcolemmal K_{ATP} channels were originally discovered in membrane patches excised from ventricular cardiomyocytes (sarcolemmal K_{ATP} channels, [1]). These channels are heteromultimers composed of, at least, two distinct subunits. The pore-forming inwardly rectifying K^+ channel core, Kir6.2, is primarily responsible for K^+ permeance, whereas the regulatory subunit, also known as the sulfonylurea receptor, or SUR2A, has been implicated in ligand-dependent channel gating [2]. More recently, it has been suggested that the sarcolemmal K_{ATP} channel protein complex may be composed of more proteins then just Kir6.2 and SUR2A, including Kir6.1 and enzymes regulating intracellular ATP levels and glycolysis [3–8]. Sarcolemmal K_{ATP} channels have been shown to play a crucial role in ischaemic preconditioning (a phenomenon when brief episodes of ischaemia/reperfusion protects the heart against myocardial infarction [9]) and myocardial resistance to ischaemia (reviewed in [10]).

Recent studies have shown that an increase in expression of SUR2A increases the number of sarcolemmal K_{ATP} channels and myocardial resistance to ischaemia/reperfusion [11]. We have found out that female gender, young age or exposure to mild hypoxia is associated with increased levels of SUR2A mRNA as well as numbers of fully assembled K_{ATP} channels and heart resistance to ischaemia/reperfusion [12–14]. When the mechanism of hypoxia-induced increase in SUR2A expression was studied, it was found that an increase in intracellular NAD triggers PI3 kinase signalling pathway leading to activation of SUR2 promoter via c-jun transcription factor [12].

It has been reported that nicotinamide-rich diet increases the intracellular levels of NAD [15]. If increase in NAD up-regulate SUR2A and K_{ATP} channels in cardiac cells, then it is possible that nicotinamide-rich diet would increase myocardial SUR2A/K_{ATP} channels and myocardial resistance to ischaemia. Therefore, we have undertaken this study to examine whether nicotinamide-rich diet would up-regulate SUR2A and increase heart resistance to ischaemia–reperfusion.

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2. Materials and methods

2.1. Nicotinamide-rich diet

C57/BL6J male mice (4–6 weeks old) were fed *ad libidum* with RPM-1 (control diet) or RPM-1 + 0.5 g/kg nicotinamide (nicotinamide-rich diet; Special Diets Services). Each mouse was fed for a week before used for experimentation. All experiments conform to the Home Office Regulations in UK. The experiments have been done under authority of Project Licences 60/3152 and 60/3925.

2.2. SUR2A mice

Generation, breeding and genotyping of these mice have previously been described in detail [11]. All experiments conform to the Home Office Regulations in UK. The experiments have been done under authority of Project Licences 60/3152 and 60/3925.

2.3. Real-time RT-PCR

Total RNA was extracted from cardiac ventricular tissue of mice using TRIZOL reagent (Invitrogen, Paisley, UK) according to the manufacturer's recommendations. Extracted RNA was further purified with RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instruction. The specific primers for mouse SUR2A, Kir6.2, Kir6.1, SUR1 and SUR2B were described in Ref. [11]. The reverse transcription (RT) reaction was carried out with ImProm-II Reverse Transcriptase (Promega, Southampton, UK). A final volume of 20 μl of RT reaction containing 4 μl of 5× buffer, 3 mM $MgCl_2$, 20 U of RNasin® Ribonuclease inhibitor, 1 U of ImProm-II reverse transcriptase, 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 0.5 μg of oligo(dT), and 1 μg of RNA was incubated at 42 °C for 1 h and then inactivated at 70 °C for 15 min. The resulting cDNA was used as a template for real-time PCR. A SYBR Green I system was used for the RT-PCR and the 25 μl reaction mixture contained: 12.5 μl of iQ™ SYBR® Green Supermix (2×), 7.5 nM each primers, 9 μl of ddH_2O, and 2 μl of cDNA. In principle, the thermal cycling conditions were as follows: an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 10 s of denaturing at 95 °C, 15 s of annealing at 56 °C, and 30 s of extension at 72 °C. The real-time PCR was performed in the same wells of a 96-well plate in the iCycler iQ™ Multicolor Real-Time Detection System (Bio-Rad, Hercules, CA). Data was collected following each cycle and displayed graphically (iCycler iQ™ Real-time Detection System Software, Version 3.0A, BioRad, Hercules, CA). Primers were tested for their ability to produce no signal in negative controls by dimer formation and then with regard to the efficiency of the PCR reaction. Efficiency is evaluated by the slope of the regression curve obtained with several dilutions of the cDNA template. Melting curve analysis tested the specificity of primers. Threshold cycle values, PCR efficiency (examined by serially diluting the template cDNA and performing PCR under these conditions) and PCR specificity (by constructing the melting curve) were determined by the same software. Each mouse cDNA sample was measured at three different quantities, and duplicated at each concentration, the corresponding no-RT mRNA sample was included as a negative control (blank [16]). The calculation of relative mRNA expression was performed as described [17]. The relative expression ratio(R) of SUR2A is calculated using equation $R = (E_K)^{\Delta CP_K(CD\text{-}NRD)}/(E_R)^{\Delta CP_R(CD\text{-}NRD)}$ (when the effect of control and nicotinamide-rich diet were assessed) or $R = (E_K)^{\Delta CP_K(WT\text{-}TG)}/(E_R)^{\Delta CP_R(WT\text{-}TG)}$ (when wild type and SUR2A transgenic mice were assessed) where E_K is the real-time PCR efficiency of a SUR2A gene transcript, E_R is the real-time PCR efficiency of GAPDH (reference) gene, ΔCP_K is the crossing point deviation of control-nicotinamide-rich diet (CD-NRD) or wild type-transgene (WT-TG) of SUR2A gene transcript while ΔCP_R is the crossing point deviation of control-nicotinamide-rich diet (CD-NRD) or wild type-transgene (WT-TG) of GAPDH gene transcript.

2.4. Heart collection and ischaemia–reperfusion injury

The heart collection and ischaemia–reperfusion injury was performed as described in details in our previous papers [18]. In brief, mice were killed by cervical dislocation (according to UK Home Office procedures), and the hearts rapidly removed and placed in ice-cold Tyrode's solution at 4 °C. The aorta was then cannulated and secured using 4–0 silk suture and the hearts were attached to a custom-made Langendorff perfusion apparatus. Hearts were perfused at a constant flow rate of 5 ml/min at 37 °C with oxygenated (95% O_2, 5% CO_2; the PO_2 in perfusate was ~600 mmHg) Tyrode's solution (in mM: NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose (Glc) 5.5, HEPES-NaOH 5.5, pH 7.4) for a stabilization period of 30 min. The heart was then subjected to 30 min of ischaemia by placing it into degassed Tyrode's (the solution was degassed with argon for 60 min and the PO_2 in this solution was ~20 mmHg) and switching off perfusion. A second 30 min reperfusion with oxygenated Tyrode's followed the ischaemia. When HMR1098 was used, it was present in the Tyrode solution throughout experimental protocol. After reperfusion, hearts were snap-frozen in liquid nitrogen and stored at −80 °C. The frozen heart was divided into approximately 5–6 transverse sections, which were weighed before staining for 1 h in 10% triphenyltetrazolium chloride (TTC) in phosphate buffer saline (PBS; both Sigma–Aldrich, Dorset, UK) at 37 °C. The stain was fixed in 4% paraformaldehyde (Sigma–Aldrich) for 30 min, following which, the tissue was photographed and the area of infarcted tissue measured using Image Analysis software [18]. Infarct sizes were calculated as $(A_1 \times W_1) + (A_2 \times W_2) + (A_3 \times W_3) + (A_4 \times W_4) + (A_5 \times W_5)$, where A is the area of infarct for the slice and W is the wt of the respective section [18].

2.5. Isolation of single cardiomyocytes

Ventricular cardiomyocytes were dissociated from the mouse using an established enzymatic procedure [19]. In brief, hearts were retrogradely perfused (at 37 °C) with medium 199, followed by Ca^{2+}-EGTA-buffered low-Ca^{2+} medium (pCa = 7), and finally low-Ca^{2+} medium containing pronase E (8 mg per 100 ml), proteinase K (1.7 mg per 100 ml), bovine albumin (0.1 g per 100 ml, fraction V) and 200 μM $CaCl_2$. Ventricles were cut into fragments in the low-Ca^{2+} medium enriched with 200 μM $CaCl_2$. Cells were isolated by stirring the tissue (at 37 °C) in a solution containing pronase E and proteinase K supplemented with collagenase (5 mg per 10 ml). The first aliquot was removed, filtered through a nylon sieve, centrifuged for 60 s (at 300–400 rpm), and washed. Remaining tissue fragments were re-exposed to collagenase, and isolation continued for 2–3 such cycles.

2.6. Experimental protocol of severe cellular hypoxia

Severe hypoxia of isolated cardiomyocytes has been performed as described [20]. Thus, cardiomyocytes were placed into Tyrode's solution (in mM: NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, HEPES-NaOH 5.5, pH 7.4), plated out on glass coverslips and paced to beat by field stimulation (parameters of the stimulation: 5–20 mV depending on cellular threshold, 5 ms, 1 Hz). Beating cardiomyocytes were perfused with Tyrode solution at a rate of 3 ml/min and, under these conditions, the partial pressure of O_2 (PO_2) in perfusate was 140 mmHg. To induce severe hypoxia, Tyrode solution was bubbled with 100% argon (PO_2 = 20 mmHg was achieved in the solution surrounding cardiomyocytes; to achieve