L-Cysteine Prevents Oxidation-Induced Block of the Cardiac Na⁺ Channel Via Interaction With Heart-Specific Cysteinyl Residues in the P-Loop Region

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The present study investigated the protective effects of L-cysteine on the oxidation-induced blockade of Na⁺ channel β-subunits, hH1 (cardiac) and hSkM1 (skeletal), expressed in COS7 cells. Na⁺ currents were recorded by the whole-cell patch clamp technique (n=3–7). L-cysteine alone blocked hH1 and hSkM1 in a dose-dependent manner, with saturating L-cysteine block at 3,000 μmol/L. Hg²⁺, a potent sulphydryl oxidizing agent, blocked hH1 with a time to 50% inhibition (Time₅₀) of 20s. Preperfusion of COS7 cells with 100 μmol/L L-cysteine significantly slowed the Hg²⁺ block of hH1 (Time₅₀ = 179 s). L-cysteine did not prevent Hg²⁺ block of hSkM1 (Time₅₀ = 37 s) or the C373Y hH1 mutant (Time₅₀ = 43 s). As for other sulfo-amino acids, homocysteine prevented the Hg²⁺ block of hH1, with the Time₅₀ (70s) being significantly smaller than that of L-cysteine, whereas methionine did not prevent the Hg²⁺ block of hH1. L-cysteine did not prevent the Cd²⁺ block of hH1. These results indicate that L-cysteine selectively acts on heart-specific Cys³⁷³ in the P-loop region of hH1 to prevent Cys³⁷³ from the oxidation-induced sulfur-Hg-sulfur bridge formation. (Circ J 2002; 66: 846–850)

Key Words: Cysteinyl residue; L-cysteine; Mercury; Na⁺ channel; P-loop region

The amino acid L-cysteine, which contains a sulphydryl group, could attenuate tissue injury caused by oxidative stress. N-acetyl-L-cysteine (NAC) has been clinically shown to prevent cardiac injuries induced by oxygen-derived free radicals and it has been experimentally proven to protect the heart from ischemic insult by opening the ATP-sensitive K⁺ channels (KATP). Although the mechanisms by which L-cysteine exerts its protective effect are still unclear, it has been reported that L-cysteine or NAC enhances the cellular thiol pool to inactivate already formed radical species and that they could directly cancel oxidative stress by reducing sulphydryl groups. Recent reports demonstrated that L-cysteine as well as NAC directly modified the properties of ionic channels, and NAC and its derivatives activate Cl⁻ conductance in epithelial cells. Tricarico and Camerino showed that L-cysteine and NAC restored the activity of KATP that had been suppressed by thimerosal-induced oxidation and the activity of KATP channels in muscle fibers of aged rats, which suggests that L-cysteine may directly reduce the oxidation of the thiol group of the channel protein, thereby modulating the channel functions. The pore-forming (P-loop) region of the Na⁺ channels β-subunit possesses one heart-specific cysteinyl residue in domain I and two conserved cysteinyl residues in domains II and IV. Recently, we studied the molecular mechanisms by which the cardiac (hH1) and skeletal type (hSkM1 or hH2) Na⁺ channel β-subunit was blocked by sulphydryl-oxidizing agents, such as divalent mercury (Hg²⁺) via oxidation of the cysteinyl residues in the P-loop region, and found that Hg²⁺ specifically reacted with the cysteinyl residues in the P-loop region of domains I, II and IV of the Na⁺ channel β-subunit to either form a sulfur-Hg-sulfur bridge or to react at a single cysteiny residue. Hence, Hg²⁺ is useful for studying the molecular mechanism of the redox regulation of Na⁺ channels. In the present study we examined the effects of L-cysteine on Hg²⁺ block of heterologous wild and mutant Na⁺ channel β-subunit expressed in COS7 cells, the aim being to (1) determine whether L-cysteine protected the Na⁺ channel β-subunit and (2) whether preperfusion of L-cysteine attenuates Hg²⁺ block of the Na⁺ channel β-subunit depending on tissue; (3) compare the effect of L-cysteine to that of other sulfur-containing amino acids, such as homocysteine and methionine; and (4) determine whether preperfusion with L-cysteine attenuated divalent cadmium (Cd²⁺) block of hH1.

Methods

Electrophysiological Recordings in Cultured Cells

The vector pRC/CMV (Invitrogen, San Diego, CA, USA) was used as the expression vector for wild type hSkM1 cDNA, wild type hH1 cDNA and mutant C373Y hH1 cDNA in which Cys³⁷³ is substituted for Tyr³⁷³, as...
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Fig 1. Effects of L-cysteine on hH1 and hSKM1. (Panel A) Representative traces of the original currents elicited with a depolarizing pulse to –20mV for 30ms from a holding potential of –120mV at 0.3Hz in the absence and presence of 100 and 1,000\textmu{M/L} L-cysteine. (Panel B) The mean ± S.E% block of hH1 and hSKM1 by L-cysteine at various concentrations (1–3,000\textmu{M/L}). The solid and hatched lines were calculated by equation (1) with the following values of parameters: Bmax = 0.49 for hH1, 0.44 for hSKM1; Kd = 307\textmu{M/L} for hH1, 166\textmu{M/L} for hSKM1; h = 0.68 for hH1, 0.90 for hSKM1. Note that L-cysteine blocked both hH1 and hSKM1 in a dose-dependent manner.

Chemical Agents Used to Modify the Sulfhydryl Groups

To modify the sulfhydryl groups of the Na\textsuperscript{+} channel protein, we used Hg\textsuperscript{2+} and Cd\textsuperscript{2+}, thiol-avid group IIb divalent cation. We used Hg\textsuperscript{2+} at a concentration of 10\textmu{M/L}, which proved sufficient to completely block both hH1 and hSKM1. Amino acids that have sulfhydryl groups, such as L-cysteine, homocysteine and methionine, were purchased from Wako (Osaka, Japan).

Data Analysis

The concentration dependence of the drug-induced block was calculated as

\[ IB/IC = Bmax/[1 + (Kd/D)^h] \]

where IB is the blocked current component at a drug concentration D, with IC being the control current in the absence of the drug. Bmax represents the maximum attainable block, Kd and h represent the dissociation constant and Hill coefficient, respectively.

Instead of measuring the dose–response curve, we measured the time when the peak amplitude of the currents after exposure to Hg\textsuperscript{2+} or Cd\textsuperscript{2+} alone reached 50% of the peak amplitude in the control and expressed it as Time50%, because it was difficult to get the dose–response curve for the Hg\textsuperscript{2+} block of the Na\textsuperscript{+} current under steady-state conditions because of the potent inhibitory effect of Hg\textsuperscript{2+} on the Na\textsuperscript{+} current.

Pooled data (n = 3–7) are presented as the mean ± SE. The statistical analysis was performed using ANOVA, and a p value <0.05 was considered as statistically significant.

Results

L-Cysteine Blocked hH1 and hSKM1 in a Dose-Dependent Manner

Panel A of Fig 1 shows that extracellular L-cysteine reduced the amplitude of either wild type hH1 or hSKM1 in a dose-dependent manner the within the range of 1–3,000\textmu{M/L} and this effect was unchanged by simple washout with normal Tyrode’s solution. Fig 1B illustrates the dependency of this blockade on the concentration of L-cysteine. According to Eq (1), the Kd and Hill coefficient for L-cysteine were 307\textmu{M/L} and 0.68 in hH1 and 166\textmu{M/L} and 0.90 in hSKM1, respectively. Interestingly, L-cysteine blocked both hH1 and hSKM1; however, the block reached a plateau at concentrations greater than 3,000\textmu{M/L}. Bmax values for hH1 and hSKM1 were 0.49 and 0.44, respectively.

We investigated whether L-cysteine affected the site at which 1,4-dithiothreitol (DTT), an authentic reducing agent, can act. After exposure to 10\textmu{M/L} DTT, L-cysteine did not reduce hH1 nor hSKM1, supporting the idea that L-cysteine acts at the same site at which reducing agents bind.

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Fig 2 shows the effects of 10\textmu{M/L} Hg\textsuperscript{2+} on hH1 currents and the attenuating effects of 100\textmu{M/L} L-cysteine on the Hg\textsuperscript{2+} block of hH1. Fig 2A,B show the amplitude of hH1 in the absence (a) and in the presence of 10\textmu{M/L} Hg\textsuperscript{2+} alone (b). Hg\textsuperscript{2+} at a concentration of 10\textmu{M/L}...
blocked hH1 immediately and completely (Time50% = 22s). Fig 2C,D show the amplitude of hH1 in the absence and in the presence of 100μmol/L L-cysteine, in that of both L-cysteine and 10μmol/L Hg2+, and in that of Hg2+ alone. L-cysteine at 100μmol/L reduced the amplitude of hH1 by 12% (b). Hg2+ reduced the amplitude of hH1 by only 20% in the presence of 100μmol/L L-cysteine (c). Following the removal of L-cysteine from the test solution, Hg2+ at a concentration of 10μmol/L blocked hH1 gradually (d and e), with the time course (Time50% = 189s) being much slower than that of Hg2+ block without preperfusion with L-cysteine (Time50% = 22s). We noticed that the reduction of hH1 during perfusion with Hg2+ alone fluctuated repeatedly between the blocked and unblocked states (d and e), until complete block was achieved. These results indicate that L-cysteine prevents Hg2+ block of hH1.

Prevention of Hg2+-Induced Na+ Channel Block by L-Cysteine is Isoform-Specific

Because Hg2+ can oxidize both the isoform-specific and the conserved cysteinyl residues in the P-loop region to block the Na+ channel β-subunit,11 we examined whether or not the protective effect of L-cysteine against Hg2+ block was isoform-dependent. Fig 3A shows the Time50% of Hg2+ block of the β-subunit of different Na+ channels after perfusion with L-cysteine. The Time50% of Hg2+ block of hH1 after perfusion with L-cysteine was significantly larger (179±7s) than either that of hSKM1 (37±13s) or that under untreated conditions. As Hg2+ is known to bind to the heart-specific cysteine residue Cys373, we used the mutant C373Y to investigate whether the effect of L-cysteine could be mediated via Cys373. The Time50% of Hg2+ block of C373Y hH1 was 43±10s, which was significantly less than that of wild hH1. These results indicate that the effect of L-cysteine is isoform-specific, suggesting that heart-specific Cys373 in the P-loop region in the Na+ channel β-subunit has a pivotal role in the selective protection of the Hg2+-induced hH1 block by L-cysteine.

Effect of Preperfusion With Homocysteine or Methionine on Hg2+ Block of hH1

We compared the effect of preperfusion with L-cysteine on Hg2+ block of hH1 to that of homocysteine or methionine. Fig 3B shows the Time50% of Hg2+ block of hH1 after perfusion with L-cysteine, homocysteine, methionine or under untreated conditions. Preperfusion with L-cysteine or homocysteine slowed the Hg2+ block of hH1. The Time50% after perfusion with L-cysteine (179±7s) or homocysteine (70±16s) was significantly larger (p<0.05) than that after perfusion with methionine (23±3s) or under untreated conditions. However the Time50% after perfusion with methionine was significantly smaller than that after perfusion with L-cysteine. Of the sulfo-amino acids tested, L-cysteine was the most effective in preventing Hg2+ block of hH1.

Fig 4. Preperfusion of COS7 cells with L-cysteine did not prevent Cd2+ block of hH1. (Panel A) Amplitude of hH1 in the presence of 100μmol/L L-cysteine, 100μmol/L Cd2+ and L-cysteine, and 300μmol/L Cd2+ and L-cysteine. Cd2+ immediately blocked hH1 in the presence of 100μmol/L L-cysteine. (Panel B) Time50% of the Cd2+-block of hH1 after preperfusion with L-cysteine obtained from 4–6 experiments. Note that preperfusion with L-cysteine did not slow Cd2+ block of hH1. (Panel C) Dose-dependent effects of Cd2+ on hH1 obtained from 5 experiments with both Kd = 30.0μmol/L and Hill coefficient = 0.89, where the ordinate is %block of hH1 and the abscissa is the concentration of Cd2+. 
L-Cysteine did not Prevent Cd²⁺ Block of hH1

Because Cd²⁺ is a thiol-avid group IIB divalent cation, as is Hg²⁺, we examined whether L-cysteine attenuated Cd²⁺ block of hH1 (Fig4). hH1 was blocked by 42% by 100 μmol/L Cd²⁺ even in the presence of L-cysteine. Then, after the removal of Cd²⁺, hH1 recovered to baseline. hH1 was completely blocked by 300 μmol/L Cd²⁺ (Panel A). Panel B shows the Times% of the hH1 block by either Hg²⁺ or Cd²⁺ after treatment with L-cysteine. The Times% of Cd²⁺ block (7±1 s) was significantly smaller than that of Hg²⁺ block (179±7 s). As shown in Panel C, Cd²⁺ blocked hH1 in a dose-dependent manner (Kd=30 μmol/L, Hill coefficient value =0.89), indicating the degree of current reduction by 100–300 μmol/L Cd²⁺ was nearly the same as in the presence of L-cysteine. This result indicates that L-cysteine did not prevent Cd²⁺ block of hH1, despite Cd²⁺ being a thiol-avid group IIB divalent cation.

Discussion

L-Cysteine Partially Blocks Na⁺ Channels

In the present study, L-cysteine blocked hH1 in a dose-dependent manner and the simple washout of L-cysteine did not restore the activity of hH1. Tracarico and Camerino9 reported that L-cysteine and NAC activated the KATP of the aged rat skeletal muscle fibers, and speculated that thiol groups, probably located on the cytoplasmic face of KATP and L-cysteine, reduced the oxidized-thiol group of KATP channel proteins to activate KATP. Their results suggest that L-cysteine reacts with ionic channels in a similar manner to a sulfhydryl-reducing agent. In the case of Na⁺ channels, DTT, the reducing agent, has been reported to reduce extracellular native S-S bonds of Na⁺ channels, which frees the SH group to block tetrodotoxin-insensitive Na⁺ channels, thereby inducing thiol-dependent redox-mediated conformational changes of Na⁺ channels pore.14,15

In the present study, after exposure to DTT, L-cysteine did not affect the hH1, indicating that the site of Na⁺ channel for L-cysteine corresponds to that for DTT. While it remains unknown whether the reduction of native S-S bonds of the Na⁺ channels can inhibit the Na⁺ current, the disulfide bridge formation in the pore region of the channel will change conductance, as described by Benitah et al.21 Thus, it is likely that L-cysteine, as well as DTT, reduces the native S-S bonds of hH1 to free SH groups and thus block hH1. The block of Na⁺ channels by L-cysteine was partial and reached a plateau at a concentration greater than 3,000 μmol/L, which is similar to the Na⁺ channel block by Zn²⁺ via creation of a subconductance state of the Na⁺ channels.16 Thus L-cysteine may produce a subconductance state via a conformational change of the channel pore; the residual currents observed at 3,000 μmol/L L-cysteine could form the subconductance state of the single Na⁺ channel.

Preperfusion With L-Cysteine Prevents Hg²⁺ Block of hH1

Preperfusion with L-cysteine significantly slowed the block of hH1 by Hg²⁺, a potent sulfhydryl oxidizing agent belonging to the group IIB of divalent cations. Kurata et al reported that DTT prevented Hg²⁺ block of cardiac Na⁺ channels, suggesting that sulfhydryl-reducing agents evoke conformational changes of the channel region containing the Hg²⁺ binding site by reducing native S-S bonds to free SH groups.14 Based on our recent experiment involving site-directed mutagenesis,1 the Hg²⁺ binding sites in the P-loop region for hH1 would be Cys373 in domain I and Cys373 in domain II and Cys1221 in domain IV. Therefore, the effect of L-cysteine on Hg²⁺ block of hH1 can be explained by the interaction of L-cysteine with cysteinyll residues in the P-loop to prevent the formation of the sulfur–Hg–sulfur bridge through the reduction of native S-S bonds of hH1. L-cysteine markedly prevented Hg²⁺ block of hH1, but not that of hSkM1. L-cysteine did not prevent the Hg²⁺ block of mutant C373YhH1. These findings suggest that the heart-specific effect of L-cysteine on Hg²⁺ block of Na⁺ channels can be attributed to the interaction of L-cysteine with the heart-specific cysteinyll residue (Cys373) in the P-loop region. P-loops are known to be flexible structures, particular in domains I and IV, suggesting the time for Cys373 to bind or unbind either Hg²⁺ or L-cysteine is very brief. Therefore, the pretreatment with L-cysteine produced a fluctuating Hg²⁺ block of hH1 from the blocked to the unblocked state. Although the precise mechanism is unknown, our results reflect the complex interactions of the channel with more than one L-cysteine molecule and the flexibility of the P-loop region involving multiple conformational transitions.17

Preperfusion with methionine did not prevent Hg²⁺ block of hH1, which also favors our hypothesis that free SH-groups of L-cysteine play a pivotal role in the effect of L-cysteine on Hg²⁺ block of hH1. Homocysteine, which has a free SH-group, prevented Hg²⁺ block of hH1, but its potency was significantly less than that of L-cysteine. Homocysteine has been reported to affect cardiac ventricular and atrial Na⁺ channels18 and we speculated that it would have less chance to interact with Cys373 of hH1 than L-cysteine.

Chemical modification experiments9,19,20 and mutant substitution experiments have indicated that Cd²⁺ and Zn²⁺, which belong to the thiol-avid group IIB divalent cations, interact with the unique sulfhydryl group of the heart Na⁺ channels (Cys373) at the same metal coordination site.21,22 We previously suggested that the mechanism of Cd²⁺ and Zn²⁺ block of Na⁺ channels differs from that of Hg²⁺ block.11 Hg²⁺ would strongly oxidize the free SH-groups to form sulfur–Hg–sulfur bridges; however, Cd²⁺ and Zn²⁺ produce a coordinate bond with the free SH groups. Therefore, L-cysteine would act on Cys373 in the P-loop region of hH1 to protect Cys373 from oxidation; however, L-cysteine did not protect Cys373 from Cd²⁺ or Zn²⁺ binding to free thiols.

Prolonged exposure of cardiac cells to free-radical generating systems has been reported to lead to a reduction of the upstroke velocity of the action potential by direct inhibition of Na⁺ channels as well as the depolarization of the membrane.23,24 This oxidant-induced inhibition of the Na⁺ channels would lead to a reduced conduction velocity, which would increase the susceptibility of the cardiac tissue to reentrant arrhythmias.25 The serum concentration of sulfhydryl groups is considered to be important for protecting the heart from ischemic-reperfusion injury.26 It has been reported that intracellular concentration of L-cysteine is 100 μmol/L, 30 μmol/L in the plasma (personal communication), and 11–73 μmol/L for the clinical range of treatment with either N-acetyl-L-cysteine or S-carboxymethyl L-cysteine, respectively. Thus, the concentration of L-cysteine (100 μmol/L) used in the present study to prevent Hg²⁺ block of Na⁺ channels was very close to the therapeutic concentration at which L-cysteine may directly act as a sulfhydryl-reducing agent on cardiac Na⁺ channels to

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prevent the oxidation-induced Na+ current block and subsequent occurrence of cardiac arrhythmia.

Acknowledgments

This study was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (0967020) (IH).

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