Fas mRNA expression and calcium influx change in H₂O₂-induced apoptotic hepatocytes in vitro

Qi-Ping Lu, Lei Tian

Abstract

AIM: To investigate the relationship between Fas gene expression and calcium influx change in peroxide-induced apoptotic hepatocytes and the possible molecular mechanism of Rxa in protecting hepatocytes.

METHODS: Single-cell Fas mRNA expression in H₂O₂-exposed L02 hepatocytes with or without treatment of Rxa, an extract from an anti-peroxidative, Radix Salviae Miltiorrhizae, was determined by all-cell patch clamp and single-cell reverse transcriptase polymerase chain reaction (RT-PCR). Transient calcium influx change ([Ca²⁺]) in the cells was evaluated with all-cell patch clamp micro-fluorescence single-cytosolic free Ca²⁺ concentration technique. Fas protein expression, early apoptotic index (annexin-V+) and membrane change in the cells were evaluated by immunohistochemistry, flow cytometry (FCM) and scan electron microscopy respectively.

RESULTS: In cells exposed to H₂O₂ for 2 h, the specific lane for Fas mRNA was vivid on electrophoresis, with increased Fas protein expression, [Ca²⁺], which was determined by immunohistochemistry, annexin-V and scan electron microscopy respectively. The cell content was aspirated and transferred into iced RT buffer and 5 u RNase was added in supernatant, and LaH group with L02 cells pre-treated with 2 mmol/L Rxa for 2 h before addition of H₂O₂, all indexes were detected 2 h after the addition of H₂O₂.

CONCLUSION: H₂O₂ induces apoptosis of L02 cells by increasing cytosolic [Ca²⁺], and inducing Fas mRNA and protein expression. Rxa protects the L02 cells from apoptosis through anti-peroxidative, inhibition of calcium overloading and prevention of the activation of cytosolic Fas signal pathway.

Key words: Hepatocytes; Apoptosis; Fas protein; Gene expression; Calcium

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with PBS. The EPC-9 all-cell patch clamp system combined with a fluorescence [Ca\textsuperscript{2+}] measuring system (HEKA, Lambert, Germany) was used to record transient living single-cell Ca\textsuperscript{2+} changes a and R (R = F1/F2; F1, F2 were cellular fluorescent rates excited at 340 nm and 380 nm, respectively). X-chart and I Cor Pro software were used to transfer Ca\textsuperscript{2+} into [Ca\textsuperscript{2+}]. Ten cells of each group were measured (Figure 1).

Figure 1 Cellular content and mRNA aspirated from a single living cell with a micropipette by a patch clamp.

**Fas protein expression**

Cells were co-incubated with 20 \( \mu \)L of 1:40 rabbit-anti-human Fas polyclonal antibody (Santa Cruz) at 4 °C overnight after routine preparation. After reaction with biotinylated goat-anti-rabbit antibody at 37 °C for 40 min, the slides were covered with SABC complex and reacted at 37 °C for another 40 min. The slides were stained with DAB and hematoxylin. Positive index (i.e., positive cells/total cells counted) of brown stained cells of the slides was calculated with the MPIAS-1000 multimedia color image analysis system.

**Measurement of early apoptotic index**

Cells were covered, washed 3 times with PBS, centrifuged at 1 000 r/min for 5 min, and incubated with annexin-V-Fluos (hepes 1 000 \( \mu \)L, 20 \( \mu \)L annexin-V, Gibco, USA) at 37 °C for 15 min, and then added with 0.4 mL hatching fluid (140 mmol/L NaCl, 5 mmol/L CaCl\textsubscript{2}, and 10 mmol/L HEPES, pH = 7.4). FACSort FCM (BD company, U.S.A) was used to quantify the annexin-V index, for which 5 mL cell supernatant was analyzed under fluorescent microscope. CELLQuest software was used to calculate the annexin-V index.

**Ultrastructure of cell membrane**

Slides plastered with L02 cells were fixed in 2.5% glutaraldehyde solution for 4 h and analyzed under a S450 scan electron microscope.

**Data analysis**

The data were presented as mean±SD for each group. SAS statistical system and F test were used to analyze the data. \( P<0.05 \) was considered statistically significant.

**RESULTS**

The baseline [Ca\textsuperscript{2+}] concentrations in L, LH and LaH groups were 142.35±28.11 nmol/L, 140.58±30.01 nmol/L, and 136.44±26.15 nmol/L, respectively, which were not significantly different. No Fas mRNA expression was observed in the control group or L2 group after the 2-h culture, the specific PCR products for Fas mRNA were not seen on the gel. The [Ca\textsuperscript{2+}], concentrations of the control group or L group at 0 and 2 h were 143.66±34.21 nmol/L, 149.77±23.20 nmol/L. Annexin-V indexes of these two groups were 4.00±0.79, 4.20±0.90 and the annexin-V cells were seldom seen under a fluorescent microscope. Comparatively, expression of Fas mRNA was vivid on electrophoresis in LH group (Figure 2). [Ca\textsuperscript{2+}] concentration and annexin-V index in this group after the 2-h treatment with H\textsubscript{2}O\textsubscript{2} were 1115.28±227.16 nmol/L and 16.18±0.72, respectively (\( P<0.01 \) vs the baseline levels). Fas protein fluorescence also significantly increased (\( P<0.01 \) vs the baseline level) and green-ring annexin-V cells were seen under a fluorescent microscope, with vesicle formation under an electron microscope (Figures 3, 4). In LaH group, there was no Fas mRNA expression (Figure 2). [Ca\textsuperscript{2+}] concentration decreased significantly to 103.56±28.92 nmol/L (\( P<0.01 \) vs the baseline level) (Figure 4), which was not significantly different from the control group. No increased Ca\textsuperscript{2+} flow was recorded with patch clamp in this group, and the Fas protein expression was lower than that in LH group (\( P<0.01 \)). Annexin-V index significantly decreased (8.92±1.44, \( P<0.01 \), vs LH group) with intact cell morphology.

![Figure 2 Fas mRNA expression as detected by RT-PCR in single L02 cells with different treatment. L, normal L02 cells as a control; LH, L02 cells with 10 \( \mu \)mol/L H\textsubscript{2}O\textsubscript{2} added in the supernatant; LaH, L02 cells pre-treated with 2 mmol/L Rxa.](image)

![Figure 3 Early phenomenon of phosphatidylinerseine PS and transformation in L02 cells of LH group.](image)

![Figure 4 Vesicle formation of L02 cells in LH group under electron microscope; (A) and normal morphology of L02 cells in LaH group (B).](image)
DISCUSSION
It is extremely important to clarify the mechanisms of lethal hepatocellular injury caused by hypoxia or ischemia and to find protective agents to improve organ preservation for liver trauma\[24,25\]. Intracellular Ca\(^{2+}\) overload is one of the factors in lethal ischemic events of cell injury\[9-15\]. Apoptosis is an important event of cell injury during ischemia/reperfusion\[16-18\]. Apoptosis is related with intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), and Fas-mediated apoptotic cell death is one of the major death processes\[19,20\]. RSM has been observed to have a cytoprotective effect on hepatocytes of rats and humans by blocking Ca\(^{2+}\) influx, but its effect on apoptosis remains unclear.

In the present study, H\(_2\)O\(_2\)-treatment increased [Ca\(^{2+}\)]\(_i\) in L02 cells and annexin -V index, and typical early apoptotic changes on cell membranes (green-ring annexin -V cells under fluorescent microscope and vesicle formation under electron microscope) were observed, with specific Fas mRNA transcription and Fas protein expression, indicating that activation of Fas death gene may mediate peroxide-triggered L02 cell apoptosis, and activation of programmed cell death may be closely related to Ca\(^{2+}\). It has been reported that increased [Ca\(^{2+}\)]\(_i\) could induce DNA fragmentation\[23,24\], but signal pathways vary with different cell types, and the relationship between Fas mRNA and [Ca\(^{2+}\)]\(_i\) change in apoptotic hepatocytes remains unclear. The cascade of type II Fas-mediated signal pathway is through mitochondria\[25\], and active metabolic hepatocytes are abundant in mitochondria. Therefore, it is inferred that increased cytoplasmic Ca\(^{2+}\) from mitochondrial trans-membrane out-flow in H\(_2\)O\(_2\)-stressed L02 cells may trigger the activation of type II Fas-mediated signal pathway. Meanwhile, stimulant-induced receptor-mediated Ca\(^{2+}\) inflow in non-excitatory hepatocytes is triggered by calcium pool exhaustion; thus H\(_2\)O\(_2\) may release endoplasmic Ca\(^{2+}\) and increase [Ca\(^{2+}\)]\(_i\), when endoplasmic Ca\(^{2+}\) is exhausted and some structures such as cyto-skeletal proteins change and connect directly or indirectly with cell membranes, which open Ca\(^{2+}\) channel and incubated extracellular Ca\(^{2+}\) inflow. Sharply increased cytoplasmic Ca\(^{2+}\) may degenerate cytoskeletal proteins and induce characteristic apoptotic changes in cell membranes. H\(_2\)O\(_2\)-induced cytoplasmic Ca\(^{2+}\) imbalance may also mediate over-expression of Fas death genes, activate death gene signal pathways and encode membrane-perforating Fas glucoprotein, which induces cells to die through apoptosis.

It was also observed in this study that pretreatment of L02 cells with Rxa could effectively inhibit the increase of [Ca\(^{2+}\)]\(_i\), and the expression of Fas mRNA and proteins and alleviate cell injury. These findings indicate that peroxide-induced activation of cytoplasmic Fas mRNA expression is Ca\(^{2+}\) dependent, and Rxa may prevent Fas mRNA expression and protect cells from apoptosis by blocking calcium. In a previous experiment we found that the role of RSM in preventing ischemia/reperfusion Ca\(^{2+}\) overload was related to its blockade of Fas mRNA expression. The anti-apoptosis and Ca\(^{2+}\) blockade of Rxa may rely on the counteraction of its phenohydroxyl radicals with oxygen in peroxide radicals and other active oxygen molecules, thus alleviating the break of cytoskeletal proteins and invasion of cell organs by oxygen, and stabilizing membrane structure. Rxa may block the interaction of the Fas trans-membrane pathway and Ca\(^{2+}\) signal pathway through stabilizing endoplasmic membrane and decreasing Ca\(^{2+}\) release and its connection with cytoplasm. Rxa may also prevent the opening of 20 nm Ca\(^{2+}\) channel on mitochondria by stabilizing mitochondrial membrane, decrease out-flow of Ca\(^{2+}\) from mitochondria, inhibit the activation of trans-mitochondrial type II Fas cascade, and prevent Fas-induced apoptosis. Our data suggest that Rxa can inhibit Fas mRNA and protein expression, prevent cell injury induced by Fas over-expression and block the signal pathway of Fas death genes, by stabilizing cell membranes, mitochondria and endoplasmic membrane, inhibit [Ca\(^{2+}\)]\(_i\) increase by blocking extracellular Ca\(^{2+}\) influx and endoplasmic Ca\(^{2+}\) release.

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