Cold preservation-warm reoxygenation increases hepatocyte steady-state Ca\(^{2+}\) and response to Ca\(^{2+}\)-mobilizing agonist

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Elimadi, Aziz, and Pierre S. Haddad. Cold preservation-warm reoxygenation increases hepatocyte steady-state Ca\(^{2+}\) and response to Ca\(^{2+}\)-mobilizing agonist. Am J Physiol Gastrointest Liver Physiol 281: G809–G815, 2001.—Although the role of Ca\(^{2+}\) in liver transplantation injury has been the object of several studies, direct evidence for alterations in intracellular Ca\(^{2+}\) homeostasis after cold preservation-warm reoxygenation (CP/WR) has never been presented. We thus investigated the effects of CP/WR on steady-state Ca\(^{2+}\) and responses to a Ca\(^{2+}\)-mobilizing agonist. Isolated rat hepatocytes were suspended in University of Wisconsin solution, stored at 4°C for 0, 24, and 48 h, and reoxygenated at 37°C for 1 h. Cytosolic Ca\(^{2+}\) was measured in single cells by digitized fluorescence videomicroscopy. CP/WR caused a significant increase in steady-state cytosolic Ca\(^{2+}\), which was inversely proportional to cell viability. Pretreatment of hepatocytes with an agent that protects mitochondrial function attenuated the increase in steady-state cytosolic Ca\(^{2+}\) and improved hepatocyte viability. Ca\(^{2+}\) responses to the purinergic agonist ATP also increased significantly as a function of cold storage time. This increase was related to an increase in the size of inositol 1,4,5-trisphosphate-sensitive Ca\(^{2+}\) stores and subsequent capacitative Ca\(^{2+}\) entry. Thus CP/WR significantly perturbs steady-state hepatocellular Ca\(^{2+}\) and responses to Ca\(^{2+}\)-mobilizing agonists, which may contribute to hepatocyte metabolic dysfunction observed after CP/WR.

Adenosine 5′-trisphosphate; endoplasmic reticulum; inositol 1,4,5-trisphosphate; mitochondria; transplantation

The hypothesis that intracellular Ca\(^{2+}\) plays a crucial role in liver transplantation injury is based essentially on the indirect observation that inhibitors of L-type voltage-dependent Ca\(^{2+}\) channels, such as nisoldipine or verapamil, improve the viability of preserved hepatocytes (45) or of liver allografts in the rat (9, 44). However, no evidence has ever been found for the presence of voltage-dependent Ca\(^{2+}\) channels on hepatocytes, either functionally (31) or at the level of mRNA (21). The effects of such Ca\(^{2+}\) channel antagonists may thus result from nonspecific beneficial actions on hepatocytes (22, 29) or from effects on Kupffer cells (20, 43). Hence, direct evidence for the perturbation of hepatocellular Ca\(^{2+}\) homeostasis after CP/WR has never been presented.

On the other hand, recent studies from our laboratory (17) and that of Kim and Southard (25) have shown that acute hypothermia alone leads to a rapid increase in steady-state intracellular Ca\(^{2+}\) in isolated hepatocytes (17). Kim and Southard (25) have shown that, after this initial increase, cytosolic Ca\(^{2+}\) subsequently decreases as cold preservation time is prolonged to 24 and 48 h (25). Both studies (17, 25) addressed perturbation of hepatocyte Ca\(^{2+}\) during simple cold storage. Information is still lacking on the effect of the overall process of liver transplantation, i.e., CP/WR (conditions that the grafted organ normally encounters), on liver cell Ca\(^{2+}\) homeostasis.

Intracellular Ca\(^{2+}\) homeostasis can be modulated by many extracellular stimuli, including ATP and related nucleotides, which have significant biological effects on many tissues and cell types such as hepatocytes. For instance, ATP has been shown to stimulate glycogen phosphorylase activity in hepatocytes, which is the rate-limiting step in liver glycogenolysis (24). ATP is also involved in intercellular signal propagation between hepatocytes and between hepatocytes and bile duct cells in the rat liver (38). Such nucleotides are released from hepatocytes themselves, from sympathetic nerve endings, as well as platelets and damaged cells during injury such as ischemia-reperfusion. Released nucleotides are known to trigger Ca\(^{2+}\) mobilization in hepatocytes (12). This mobilization consists of...
two phases, namely \( \text{Ca}^{2+} \) release from intracellular stores and \( \text{Ca}^{2+} \) influx across the plasma membrane (42). Should \( \text{Ca}^{2+} \) homeostatic mechanisms be affected by CP/WR, alterations in \( \text{Ca}^{2+} \)-dependent hepatocyte functions such as metabolism and bile secretion may ensue and form, at least in part, the basis of primary graft dysfunction.

We thus sought to evaluate the impact of long-term CP/WR on hepatocyte \( \text{Ca}^{2+} \) homeostasis, particularly at the level of steady-state intracellular \( \text{Ca}^{2+} \), and of \( \text{Ca}^{2+} \) responses to the purinergic agonist ATP. We used an in vitro model that appropriately mimics CP/WR in liver cells (13, 17, 18, 39–41). We report that CP/WR significantly increases steady-state hepatocellular \( \text{Ca}^{2+} \). Responses to the \( \text{Ca}^{2+} \)-mobilizing agent ATP are also increased, and this was related to an increase in the size of intracellular inositol 1,4,5-trisphosphate (IP\(_3\))-sensitive \( \text{Ca}^{2+} \) stores and in the subsequent capacitative \( \text{Ca}^{2+} \) entry.

MATERIALS AND METHODS

**Hepatocyte isolation.** Animals used in this study were treated in accordance with the guidelines of the Canadian Council on the Care of Animals, and all experimental protocols were approved by our university’s ethics committee.

Hepatocytes were isolated from the livers of fed male Sprague-Dawley rats weighing 200–250 g (Charles River Laboratories, St. Constant, QC, Canada) by a variation of the classical collagenase perfusion as described previously (19). Hepatocytes were purified by centrifugation on a Percoll gradient. The initial viability of hepatocyte preparations averaged 90% as indicated by trypan blue exclusion. Isolated rat hepatocytes were then suspended in University of Wisconsin (UW) solution and stored undisturbed in sealed 50 ml conical tubes at 4°C for 24 or 48 h. At the end of the cold preservation period, UW solution was aspirated and hepatocytes washed in cold Williams’ medium E at 37°C for 1 h. The cells were then incubated at 37°C in the stirred thermostated cuvette of a SPEX model CMT-111 spectrofluorometer (Rayonics Scientific, St. Laurent, QC, Canada) in a “cytosol-like” medium (120 mM KCl, 10 mM NaCl, 1 mM KH\(_2\)PO\(_4\), 1 mM MgCl\(_2\), 30 mM HEPES, and 1 mM ATP, pH 7.4, at 37°C). This medium also contained an ATP-generating system (25 mM creatine phosphate, 25 U/ml creatine kinase, and 5 mM sodium pyrophosphate) and 10 \( \mu \)M FCCP, a mitochondrial decoupler, to eliminate the participation of mitochondria in intracellular \( \text{Ca}^{2+} \) handling during this experiment. Plasma membranes were permeabilized by adding 50 \( \mu \)g/ml saponin. Permeabilized cells were washed once in the absence of saponin, gently pelleted, and resuspended in 1 ml cytosol-like medium without saponin at a density of 10\(^7\) cells/ml, as previously described by Missiaen and co-workers (32). IP\(_3\)-induced \( \text{Ca}^{2+} \) mobilization was carried out in the presence of 10 \( \mu \)M fura 2-free acid. IP\(_3\) was added at concentrations of 1 to 50 \( \mu \)M. Fluorescence experiments were carried out using excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. Fluorescent signals were captured by photomultiplier tubes, and the data were analyzed using the software supplied by SPEX.

**Reagents.** All reagents were of the highest quality available and obtained from Sigma-Aldrich Chemical (Mississauga, ON, Canada). Collagenase (type D) was obtained from Roche Diagnostics (Laval, QC, Canada).

**Statistical analysis.** Each experiment was performed with at least four to eight separate hepatocyte preparations. Values are presented as means \( \pm \) SE of the indicated number of cells. Statistical analysis was performed using one- or two-way ANOVA as appropriate. \( P < 0.05 \) was considered statistically significant.

RESULTS

**Effect of cold preservation followed by warm reoxygenation on hepatocyte steady-state intracellular \( \text{Ca}^{2+} \) and cell viability.** Steady-state intracellular free \( \text{Ca}^{2+} \) concentration of control cells under our experimental conditions was 95 \( \pm \) 3 nM (\( n = 151 \) cells). As shown in Fig. 1, when hepatocytes were preserved for 24 and 48 h in UW followed by 1 h of warm reoxygenation, steady-state intracellular \( \text{Ca}^{2+} \) concentration increased significantly to 224 \( \pm \) 11 and 286 \( \pm \) 21 nM, respectively (\( n = 100 \) and 130 cells, respectively; \( P < 0.05 \) for either group compared with control unstored.
to the phosphatidylinositol-Ca\textsuperscript{2+} signaling pathway and to increase cytosolic free Ca\textsuperscript{2+} concentration in rat hepatocytes (12). ATP (100 μM) induced a typical biphasic Ca\textsuperscript{2+} response in all cells. Responses were quantified by measuring the area under the Ca\textsuperscript{2+} vs. time curve (AUC) as shown in Fig. 2. Reoxygenating the hepatocytes after preserving them at 4°C for 24 and 48 h significantly increased the total amount of Ca\textsuperscript{2+} mobilized by ATP (Fig. 2, A and C). We then used EGTA (4 mM), a known Ca\textsuperscript{2+} chelator, to eliminate the Ca\textsuperscript{2+} influx phase of the ATP-induced Ca\textsuperscript{2+} response and hence to isolate Ca\textsuperscript{2+} release from intracellular stores. CP/WR also significantly increased the quantity of Ca\textsuperscript{2+} mobilized by ATP in the presence of EGTA (Ca\textsuperscript{2+}-free conditions, Fig. 2, B and C), indicating a greater release from internal stores. In physiological conditions, the rise in Ca\textsuperscript{2+} response to ATP was dependent on cold preservation time \( (P < 0.05) \), for 24 and 48 h vs. unstored controls (0 h) as well as between 24- and 48-h preservation groups). On the other hand, in Ca\textsuperscript{2+}-free conditions, the increase in ATP response (AUC) in relation to unstored controls was similar for 24- and 48-h groups. This was confirmed by two-way ANOVA, which uncovered a significant interaction between extracellular Ca\textsuperscript{2+} status and cold preservation time \( (P < 0.05) \). Our results further indicate that the capacitative Ca\textsuperscript{2+} entry that follows mobilization of internal Ca\textsuperscript{2+} stores was also increased as a function of cold preservation time as indicated by the significant interaction \( (P < 0.05) \) of the two-way ANOVA.

**Effect of CP/WR on IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} pools.** The Ca\textsuperscript{2+} mobilized from intracellular stores by G\textsubscript{q}-coupled receptor agonists, such as ATP, comes mainly from IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} pools (42). The results of Fig. 2, B and C, obtained in Ca\textsuperscript{2+}-free conditions thus prompted us to investigate whether the potency (EC\textsubscript{50}) of IP\textsubscript{3} or the size (E\textsubscript{max}) of IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} pools was affected by CP/WR. For this purpose, we used permeabilized hepatocytes stimulated with exogenous IP\textsubscript{3}. Figure 3 shows the effects of increasing concentrations of IP\textsubscript{3} on Ca\textsuperscript{2+} mobilization from IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} pools in hepatocytes permeabilized after being submitted to cold preservation followed by warm reoxygenation. **Table 1.**

<table>
<thead>
<tr>
<th>Preservation Time, h</th>
<th>Cell Viability, % Control</th>
<th>+10 μM MIBG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>84.6 ± 1</td>
<td>82.6 ± 1.8</td>
</tr>
<tr>
<td>24</td>
<td>64.7 ± 0.5*</td>
<td>70.7 ± 1.5†</td>
</tr>
<tr>
<td>48</td>
<td>56.9 ± 1.5*</td>
<td>63.7 ± 1.7†</td>
</tr>
</tbody>
</table>

Values are means ± SE from at least 4 different cell preparations. Hepatocyte preparations were treated with 10 μM m-iodobenzylguanidine (MIBG) or the equivalent volume of saline solution, preserved in cold University of Wisconsin (UW) solution at 4°C for 24 or 48 h, and then reoxygenated at 37°C for 1 h. Hepatocyte viability was measured by means of trypan blue exclusion (similar relationships and statistical significance were obtained with lactate dehydrogenase release, data not shown). *P < 0.05, significantly different from unstored control (0 h); †P < 0.05, significantly different from respective untreated cells (saline vehicle of MIBG).

**Table 1. Effect of MIBG on hepatocyte viability after cold preservation followed by warm reoxygenation**

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**Protection of mitochondrial functions attenuates increase in steady-state intracellular Ca\textsuperscript{2+} after CP/WR.** Mitochondria have been widely implicated in both warm and cold ischemia-reperfusion injury (10, 11, 37) and in intracellular Ca\textsuperscript{2+} homeostasis (36). Hepatocytes were thus incubated with MIBG, a pharmacological agent shown (25) to protect mitochondrial function under similar conditions, the rise in Ca\textsuperscript{2+} response to ATP was a significant decrease in cell viability in unstored control cells. However, when hepatocytes were preserved for 24 and 48 h at 4°C followed by 1 h of warm reoxygenation in the continued presence of m-IGB, the rise of steady-state intracellular Ca\textsuperscript{2+} observed in untreated cells was significantly attenuated. Two-way ANOVA uncovered a significant interaction \( (P < 0.05) \) between MIBG treatment and cold preservation time. This indicates that the protective effect of MIBG was greater in the 48-h than in the 24-h preserved group. Interestingly, treatment of isolated hepatocytes with MIBG was also associated with a modest yet statistically significant improvement in hepatocyte viability after CP/WR (Table 1). Also, similarly to results on steady-state Ca\textsuperscript{2+}, MIBG did not have any effect on cell viability in unstored control cells.

**Effect of CP/WR on hepatocyte Ca\textsuperscript{2+} responses to the purinergic agonist ATP.** ATP is known to act on P\textsubscript{2Y2} receptors (previously known as P\textsubscript{2U} receptors) coupled with the phosphatidylinositol-Ca\textsuperscript{2+} signaling pathway and to increase cytosolic free Ca\textsuperscript{2+} concentration in rat hepatocytes (12). ATP (100 μM) induced a typical biphasic Ca\textsuperscript{2+} response in all cells. Responses were quantified by measuring the area under the Ca\textsuperscript{2+} vs. time curve (AUC) as shown in Fig. 2. Reoxygenating the hepatocytes after preserving them at 4°C for 24 and 48 h significantly increased the total amount of Ca\textsuperscript{2+} mobilized by ATP (Fig. 2, A and C). We then used EGTA (4 mM), a known Ca\textsuperscript{2+} chelator, to eliminate the Ca\textsuperscript{2+} influx phase of the ATP-induced Ca\textsuperscript{2+} response and hence to isolate Ca\textsuperscript{2+} release from intracellular stores. CP/WR also significantly increased the quantity of Ca\textsuperscript{2+} mobilized by ATP in the presence of EGTA (Ca\textsuperscript{2+}-free conditions, Fig. 2, B and C), indicating a greater release from internal stores. In physiological conditions, the rise in Ca\textsuperscript{2+} response to ATP was dependent on cold preservation time \( (P < 0.05) \), for 24 and 48 h vs. unstored controls (0 h) as well as between 24- and 48-h preservation groups). On the other hand, in Ca\textsuperscript{2+}-free conditions, the increase in ATP response (AUC) in relation to unstored controls was similar for 24- and 48-h groups. This was confirmed by two-way ANOVA, which uncovered a significant interaction between extracellular Ca\textsuperscript{2+} status and cold preservation time \( (P < 0.05) \). Our results further indicate that the capacitative Ca\textsuperscript{2+} entry that follows mobilization of internal Ca\textsuperscript{2+} stores was also increased as a function of cold preservation time as indicated by the significant interaction \( (P < 0.05) \) of the two-way ANOVA.

**Effect of CP/WR on IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} pools.** The Ca\textsuperscript{2+} mobilized from intracellular stores by G\textsubscript{q}-coupled receptor agonists, such as ATP, comes mainly from IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} pools (42). The results of Fig. 2, B and C, obtained in Ca\textsuperscript{2+}-free conditions thus prompted us to investigate whether the potency (EC\textsubscript{50}) of IP\textsubscript{3} or the size (E\textsubscript{max}) of IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} pools was affected by CP/WR. For this purpose, we used permeabilized hepatocytes stimulated with exogenous IP\textsubscript{3}. Figure 3 shows the effects of increasing concentrations of IP\textsubscript{3} on Ca\textsuperscript{2+} mobilization from IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} pools in hepatocytes permeabilized after being submitted to cold preservation followed by warm reoxygenation.
As seen from both Fig. 3 and the values presented in Table 2, the $E_{\text{max}}$ of IP$_3$-sensitive Ca$^{2+}$ pools increased in a statistically significant manner in both groups of cold-preserved cells (24 or 48 h) compared with unstored controls. Interestingly, and in accordance with the results shown in Fig. 2, B and C, there was no difference between the 24- and 48-h groups at the level of the increased $E_{\text{max}}$ for IP$_3$. On the other hand, the $EC_{50}$ of IP$_3$ was not affected by our experimental conditions (Table 2).

**DISCUSSION**

In the current study, we measured hepatocyte intracellular Ca$^{2+}$ concentration directly at the single cell level in experimental conditions mimicking the CP/WR of liver transplantation. We present, for the first time, direct evidence showing that the intracellular Ca$^{2+}$ homeostasis of isolated hepatocytes is altered when they are submitted to CP/WR. Indeed, steady-state intracellular Ca$^{2+}$ concentration is increased after CP/WR of the hepatocytes, and this increase is accom-

### Table 2. Effect of cold preservation followed by warm reoxygenation on potency and efficacy of IP$_3$

<table>
<thead>
<tr>
<th>Preservation Time, h</th>
<th>$EC_{50}$, $\mu$M IP$_3$</th>
<th>$E_{\text{max}}$, nM Ca$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.4 ± 2.1</td>
<td>300 ± 34</td>
</tr>
<tr>
<td>24</td>
<td>11.3 ± 4.6</td>
<td>497 ± 54*</td>
</tr>
<tr>
<td>48</td>
<td>8.8 ± 0.7</td>
<td>523 ± 63*</td>
</tr>
</tbody>
</table>

Values represent means ± SE from at least 4 different cell preparations. Hepatocytes were preserved in cold UW solution at 4°C for 0, 24, and 48 h and then reoxygenated at 37°C for 1 h. Hepatocytes were then permeabilized with saponin, and Ca$^{2+}$ was mobilized from the IP$_3$-sensitive calcium pools by addition of different concentration of synthetic IP$_3$. Ca$^{2+}$ was measured in cell suspensions using fura 2 fluorescence as described in MATERIALS AND METHODS. Efficacy ($E_{\text{max}}$) and potency $EC_{50}$ values were derived from the best nonlinear Michaelian fit of the data means for each group determined with a commercially available software (Micropharm INSERM; 46). *$P < 0.05$, significantly different from unstored control (0 h).
panied by a decrease in hepatocyte viability. Consistent with our observation, an increase in basal intracellular Ca\textsuperscript{2+} associated with a decrease in cell viability has also been reported (6) during oxidative stress in rat hepatocytes. This brings forth the interesting possibility that production of reactive oxygen species may be implicated in mediating the increase in steady-state cytosolic Ca\textsuperscript{2+} in hepatocytes subjected to CP/WR.

Recent research (37) has established that liver mitochondria are seriously damaged during CP/WR. Furthermore, mitochondria have been shown (3, 36) to participate actively in intracellular Ca\textsuperscript{2+} homeostasis. We thus used MIBG as a pharmacological tool to assess the implication of mitochondria in the observed effects of CP/WR on hepatocyte cytosolic Ca\textsuperscript{2+}. This agent is known to prevent mitochondrial Ca\textsuperscript{2+} efflux in isolated hepatocytes without signs of cytotoxicity (23). MIBG has recently been shown (25) to protect mitochondrial functions altered by cold preservation. In the present study, pretreatment of hepatocytes with MIBG partially but significantly attenuated the elevation in steady-state intracellular Ca\textsuperscript{2+} observed when hepatocytes were subjected to CP/WR. No effect of this agent was observed on either steady-state intracellular Ca\textsuperscript{2+} or on cell viability in unstored control cells (0 h). This confirms that MIBG has no effect on hepatocellular Ca\textsuperscript{2+} and viability under normal physiological conditions. Interestingly, pretreatment of the hepatocytes with MIBG slightly but significantly improved cell viability after CP/WR. This is in accordance with the findings of Kim and Southard (25) who showed that MIBG ameliorates both mitochondrial functions and ATP regeneration in hepatocytes exposed to long-term cold preservation. Cyclosporin A, which possesses the same molecular mechanism as MIBG at the mitochondrial level, has recently been reported (16) to exert similar protective effects against reoxygenation injury in isolated rat cardiomyocytes. Both agents have long been known to inhibit mitochondrial permeability transition (MPT) (47). Furthermore, MPT has been shown to be the hallmark of mitochondrial damage observed in cold preserved-warm perfused isolated rat liver (27) as well as in warm ischemia-reperfusion injury (11). Our results thus support the notion that alterations in mitochondrial function after CP/WR are caused by opening of the MPT pore. This leads to the release of Ca\textsuperscript{2+} from mitochondria, thus reducing their Ca\textsuperscript{2+}-buffering capacity and increasing intracellular Ca\textsuperscript{2+} under these pathological conditions. Indeed, treatment of hepatocytes with the mitochondrial protector and MPT inhibitor MIBG enhances mitochondrial function after CP/WR. Consequently, mitochondrial Ca\textsuperscript{2+} storage capacity is restored, thus helping to partly prevent the rise of steady-state intracellular Ca\textsuperscript{2+} observed after CP/WR.

To gain further insight into the perturbations of hepatocellular Ca\textsuperscript{2+} homeostasis after long-term CP/WR, we investigated the effects of these conditions on the Ca\textsuperscript{2+} responses to the purinergic agonist ATP. The results of the present study clearly demonstrate that compound Ca\textsuperscript{2+} responses to ATP (as evaluated by AUC analysis) are increased as a function of cold preservation time. As mentioned previously, these compound responses are generally composed of two phases, namely Ca\textsuperscript{2+} release from intracellular stores and Ca\textsuperscript{2+} influx across the plasma membrane (42). We therefore used EGTA-induced chelation of extracellular Ca\textsuperscript{2+} and show that the increase in AUC, observed in response to ATP after CP/WR, was partly due to an increase in agonist-induced Ca\textsuperscript{2+} release from intracellular stores.

ATP is known to stimulate IP\(_3\) formation, which in turn activates Ca\textsuperscript{2+} release from the endoplasmic reticulum, generally considered as the principal IP\(_3\)-sensitive Ca\textsuperscript{2+} store (12). We have therefore used permeabilized hepatocytes challenged with exogenous IP\(_3\) to examine the effect of CP/WR on the sensitivity and storage capacity of IP\(_3\)-sensitive Ca\textsuperscript{2+} stores. We found that the E\(_{max}\) of the IP\(_3\)-sensitive Ca\textsuperscript{2+} pools increased with the time of preservation, whereas the EC\(_{50}\) of IP\(_3\) in mobilizing Ca\textsuperscript{2+} was not altered. Our results thus suggest that responses to any Ca\textsuperscript{2+}-mobilizing agonist (such as \(\alpha\)-adrenergic agonists angiotensin II or vasoressin) will be expected to increase as a function of cold preservation time for a given amount of IP\(_3\) produced.

Recent studies (28) have shown that IP\(_3\)-sensitive Ca\textsuperscript{2+} pools can be influenced by intracellular Ca\textsuperscript{2+} level. An increase in steady-state intracellular Ca\textsuperscript{2+} leads to an increase in the size of IP\(_3\)-sensitive Ca\textsuperscript{2+} pools, whereas a decrease in cytosolic Ca\textsuperscript{2+} reduces the size of these pools. It is therefore possible that the increase in steady-state Ca\textsuperscript{2+} that we observed after long-term CP/WR leads to a greater capacity of IP\(_3\)-sensitive Ca\textsuperscript{2+} pools to store Ca\textsuperscript{2+} and hence to a greater response to Ca\textsuperscript{2+}-mobilizing agonists. In addition, Missiaen et al. (32) demonstrated that increases in cytosolic Ca\textsuperscript{2+} sensitize IP\(_3\)-sensitive pools to IP\(_3\), so that the increased steady-state Ca\textsuperscript{2+} after CP/WR may also have contributed to enhance the response to Ca\textsuperscript{2+}-mobilizing agonists.

Finally, our results suggest that capacitative Ca\textsuperscript{2+} entry was also increased in a preservation time-dependent manner (the part of the ATP response inhibited by EGTA). Indeed, the Ca\textsuperscript{2+} entry phase of the response to Ca\textsuperscript{2+}-mobilizing agonists is known to be regulated by the Ca\textsuperscript{2+} content of the stores, a process referred to as store-operated or capacitative Ca\textsuperscript{2+} entry (33). It is thus conceivable that part of the rise in cytosolic Ca\textsuperscript{2+} observed after CP/WR (possibly that part not prevented by MIBG) was related to an increased tonic influx of Ca\textsuperscript{2+} from the extracellular milieu through conductive pathways.

It is well known that the endoplasmic reticulum plays an important role in Ca\textsuperscript{2+} storage and signaling and in the folding of newly synthesized membrane and secretory proteins, reactions that are strictly Ca\textsuperscript{2+} dependent (for review, see Ref. 34). Therefore, a perturbation of the IP\(_3\)-sensitive Ca\textsuperscript{2+} stores might alter these functions and participate in the functional disorders of CP/WR. In agreement with this assumption,
Pinton et al. (35) recently found that overexpression of the protooncogene Bcl-2 reduces both the loading of intracellular Ca\textsuperscript{2+} stores and the capacitative Ca\textsuperscript{2+} influx. In addition, overexpression of the Bcl-2 transgene protects mouse liver and cardiac cells from ischemia-reperfusion injury (5, 7). Thus conditions with a reduced size of IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} pools are associated with less ischemia-reperfusion injury. Conversely, a weakness in the functions of the Bcl-2 family of proteins (for instance, reduced inhibition of MPT; 14) could participate in the deleterious effects of CP/WR injury to Ca\textsuperscript{2+} of livers undergoing transplantation.

In conclusion, we find that in hepatocytes, CP/WR causes a significant increase in steady-state cytosolic Ca\textsuperscript{2+} concentration that is associated with a decrease in cell viability. The elevation in the steady-state Ca\textsuperscript{2+} can be significantly attenuated by protecting mitochondrial functions. Moreover, responses to the purinergic agonist ATP are also increased, and this is due to an increase in the size of IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} pools as well as in capacitative Ca\textsuperscript{2+} entry. This alteration in both the steady-state intracellular Ca\textsuperscript{2+} and in the response to Ca\textsuperscript{2+}-mobilizing agonists might contribute to the primary graft dysfunction observed after CP/WR injury of livers undergoing transplantation.

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