Concomitant Administration of Mannitol and N-Acetylcysteine for the Prevention of Lung Reperfusion Injury

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Background: Mannitol (MN) and N-acetylcysteine (NAC) are partially successful in preventing lung reperfusion injury after liver ischemia reperfusion (IR). Their concomitant administration might enhance the individual effects of each.

Methods: Rat isolated livers were perfused with Krebs-Henseleit solution (controls) or made globally ischemic (IR) for 2 hours. Separately isolated lungs were paired with livers and each pair was reperfused in-series for 15 minutes. During reperfusion, eight groups were treated with Krebs containing two low and two high doses of MN and/or NAC; one group received no treatment.

Results: The tested lung parameters were unchanged in all control groups. Pulmonary perfusion or ventilatory pressures, weight gain and bronchoalveolar lavage volume increased by 30 to 70% of baseline in the nontreated IR-paired lungs and in the only IR-MN 0.44 mmol (weight/body weight) treated lungs but remained preserved by the two higher monotherapies (MN 0.55 mmol and NAC 0.37 mmol) and by the four bitherapies. The reduced glutathione content in all lung tissue subgroups treated by the bitherapies was higher by 63 to 124% of the corresponding monotherapy values. Xanthine oxidase activity in the bitherapies-treated IR-lungs decreased 1.5 to twofold compared with the corresponding monotherapies.

Conclusions: Co-administration of MN and NAC augments the amount of lung protection afforded by each drug individually and enhances their antioxidant potentials.

Key Words: Ischemia-reperfusion, Liver, Lung, Mannitol, N-acetylcysteine.
Isolated Perfused Liver and Lung Preparation

A laparotomy was performed and the liver was isolated and perfused with hemoglobin-free, oxygenated, modified Krebs-Henseleit solution (Krebs) via the portal vein and the suprahepatic inferior vena cava as previously described. The liver was then placed in a water-insulated environmental chamber (constant temperature 37°C) and perfused at a rate of 4 mL/min/g liver ideal weight (42–48 mL/min) or as required to maintain a normal exiting pH in the perfusate during stabilization.

Lungs from different animals were tracheostomized and ventilated (95% air-5% CO₂, 1 mL/100 g body weight tidal volume and 40 breaths/min) and perfused via the main pulmonary artery and left atrium with Krebs containing 5% (wt/vol) bovine serum albumin. The addition of albumin to the Krebs solution did not interfere with any of the biochemical analyses. End-expiratory pressure was maintained at +2 cm H₂O and pulmonary venous pressure was kept constant at +2.5 cm H₂O. The lungs were then suspended from a force displacement transducer (Grass Instruments Co., Quincy, Mass.) within an acrylic, water-insulated (37°C) environmental chamber. The lung perfusion rate ranged between 15 to 18 mL/min.

Drug Protocol

The postliver reperfusate was reconstructed before entering the lungs in the appropriate groups (see below) to contain a high or a low dose of the experimental drugs (weight/rat body weight, mmol). MN (Baxter, Ashdod, Israel) was administered either alone or in combination with a high or low dose of NAC (BF Goodrich Diamalt GmbH, Raubling, Germany); the doses of NAC could also be administered singly (see below). The solutions were always isosmotic. A pilot study in our laboratory had found that doses less than 0.44 mmol MN and 0.25 mmol NAC were totally ineffective.

Experimental Protocol

Figure 1 illustrates the experimental protocol. Following 30 minutes of stabilization (Fig. 1A), an isolated liver was perfused for 2 hours or made globally ischemic (flow arrest). Toward the end of this period, one lung was isolated and stabilized for 30 minutes (Fig. 1B). At this point, each liver was connected in-series to a lung, so that the hepatic effluent was directed into the pulmonary circulation through a heated membrane oxygenator (Fig. 1A,B). The pair of organs was perfused together for 15 minutes after which the liver was removed from the circuit and the lung was left to recirculate for additional 45 minutes with the effluent that had been accumulated during the earlier 15-minute serial reperfusion period (B). While the liver was always perfused in a single-pass mode, the lung was perfused mainly in a closed-loop recirculation mode.

There were nine experimental study groups with a total of 12 replicates each. Half (n = 6) of the livers in each group underwent 2 hours of perfusion (control subgroups) and the other half underwent ischemia (IR subgroups), thus yielding 18 subgroups. Each liver had a normal lung attached to it during reperfusion. The nine study groups underwent the...
Table 1 Experimental Drugs Protocol

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
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<tbody>
<tr>
<td>No treatment</td>
<td>2-hr liver normal perfusion-paired lung subgroups (n = 6/subgroup); no drug added into the Krebs</td>
</tr>
<tr>
<td>M44</td>
<td>Control- or IR-liver-paired lung subgroups (C-M44, IR-M44) perfused with Krebs-added mannitol (MN) 0.44 mmol</td>
</tr>
<tr>
<td>M55</td>
<td>Control- or IR-liver-paired lung subgroups (C-M55, IR-M55) perfused with Krebs-added mannitol (MN) 0.55 mmol</td>
</tr>
<tr>
<td>N25</td>
<td>Control- or IR-liver-paired lung subgroups (C-N25, IR-N25) perfused with Krebs-added N-acetyl-L-cysteine (NAC) 0.25 mmol</td>
</tr>
<tr>
<td>N37</td>
<td>Control- or IR-liver-paired lung subgroups (C-N37, IR-N37) perfused with Krebs-added N-acetyl-L-cysteine (NAC) 0.25 mmol</td>
</tr>
<tr>
<td>M44N25</td>
<td>Control- or IR-liver-paired lung subgroups (C-M44N25, IR-M44N25) perfused with Krebs-added mannitol (MN) 0.44 mmol plus N-acetyl-L-cysteine (NAC) 0.25 mmol</td>
</tr>
<tr>
<td>M44N37</td>
<td>Control- or IR-liver-paired lung subgroups (C-M44N37, IR-M44N37) perfused with Krebs-added mannitol (MN) 0.44 mmol plus N-acetyl-L-cysteine (NAC) 0.37 mmol</td>
</tr>
<tr>
<td>M55N25</td>
<td>Control- or IR-liver-paired lung subgroups (C-M55N25, IR-M55N25) perfused with Krebs-added mannitol (MN) 0.55 mmol plus N-acetyl-L-cysteine (NAC) 0.37 mmol</td>
</tr>
<tr>
<td>M55N37</td>
<td>Control- or IR-liver-paired lung subgroups (C-M55N37, IR-M55N37) perfused with Krebs-added mannitol (MN) 0.55 mmol plus N-acetyl-L-cysteine (NAC) 0.37 mmol</td>
</tr>
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Every group (n = 12/group) was divided into two subgroups (n = 6/each), one consisting of lungs paired to control and one to IR livers. Each lung preparation was separately stabilized for 30 min before being paired to a liver and perfused with its effluent.

The following protocol: lungs not treated (i.e. Krebs not containing any drug) in one group whereas the lungs in the other eight groups were treated starting at the reperfusion phase (of either control or IR livers) with one of the drug protocols via a side arm attached to the postliver prelung circuit. These eight protocols consisted of four monotherapies (two MN doses [one low and one high dose] and two NAC doses [one low and one high dose]) and four bitherapies (combinations of the four monotherapies) (Table 1).

Data Collection

Samples for laboratory tests were taken every 15 minutes, while physiologic variables were recorded continuously (see below). Additional laboratory sampling time points were at 1, 5, and 10 minutes during the in-series reperfusion period.

Biochemical Analyses

Following previous demonstrations that increased circulating hepatocellular compounds and enzymes was indicative of liver damage induced by a 2-hour IR that was later associated with secondary organ damage, the activity of aspartate aminotransferase (AST) in the bronchoalveolar lavage (BAL) was assessed in duplicate to mark liver damage, using a commercial kit of Roche–Boehringer (Mannheim GmbH Diagnostic, Mannheim, Germany) and a Hitachi 747 (Tokyo, Japan) analyzer. The determination of the total XO + XDH (the oxidized and reduced forms) activity was aimed at indirectly quantifying the potential detrimental activity of ROS. Total XO was analyzed in the BAL fluid following the method of Hashimoto and as previously described.

Portions of lung tissue were also used to determine XO. Glutathione in its reduced form (GSH) was measured in the tissue, using a specific assay kit (Calbiochem 354102 San Diego, Calif.).

Lung Physical Data

These parameters comprised pulmonary perfusion pressure, intra-experimental lung weight gain, peak ventilatory pressure, and BAL volume and content. Pressures were measured directly via pressure transducers and continuously recorded on a physiologic recorder (Grass Model 7D polygraph, Grass Instruments Co., Quincy, Mass.). At the end of the experiment, the lung’s airways were gently flushed three times with 1 mL of warm saline through the trachea, and the fluid was left to drain out passively. A portion from each lung was then weighed and dried at 80°C for 7 days so that the wet-weight-to-dry-weight ratio (WDR) could be calculated. This index was found to linearly represent alveolocapillary membrane dysfunction and was used in the current investigation since the model mimicked clinical lung injury associated with fluid movement into the lung tissue.

Statistics

The data variables were summarized as means ± SEM. At each time point, a post hoc analysis was done using a two-way analysis of co-variance (ANCOVA) with repeated measures to compare changes within lung groups over time. The comparisons between group means were carried out by the Student Newman-Keuls test; enzymes concentrations in the BAL or in the lung tissue were analyzed by the same test. The level of significance was set at *p < 0.05.

RESULTS

Most of the values for the parameters in the control-treated subgroup of lungs were almost identical to those in the nontreated subgroup. The data of all these lungs, therefore, are reported cumulatively and are collectively referred to as “controls,” unless otherwise specified.
Biochemistry of the Double-Organ Model

Both the fractionated BAL indices (i.e., fluid volume retrieved after each flush and its contents, data not shown) and the total BAL volumes retrieved from the control lung subgroups were significantly (*p < 0.05) lower compared with those obtained in the IR-0 and the two low-dose (IR-M44, IR-N25) monotherapy subgroups. The higher two monotherapies (IR-M55, IR-N37) and all four bitherapy subgroups of lungs (IR-M44N25, IR-M55N25, IR-M44N37, and IR-M55N37) yielded a similar total volume as that of the controls (Table 2). A similar trend was observed for the volume-calculated solutes, i.e. a similar total volume as that of the controls (Table 2). A similar

Figure 2 (upper plane) shows the total XO activity in the tissue that was similarly low in the various control subgroups. It increased (*p < 0.01) to levels above those of the controls in the IR-0 and, to a significantly lesser degree, in the four monotherapy-treated lungs. Among these latter four, the higher dose monotherapies were associated with less XO activity than the lower dose ones. The activity in the four-bitherapy subgroups was low when compared with the IR-0 lungs and, more importantly, in comparison to all monotherapy-treated subgroups, with the activity becoming significantly (*p < 0.05) lower in three of the corresponding bitherapy-control counterparts.

Tissue GSH content in the various subgroups is also displayed in Fig. 2 (lower plane). The content in all the control monotherapy-treated lungs remained almost unchanged from that of the nontreated controls, except for the N37 lungs in which the GSH value was 72% higher than that of the control lungs. The bitherapy-treated control lungs contained up to 218% more GSH than the nontreated ones, equivalent to there being 116% more GSH than the sum of GSH assessed in the pair of monotherapies that composed each of these bitherapies. In the various IR-paired-treated lungs, the GSH values were all lower by 26 to 38% of their control counterparts. The maximum GSH value in any monotherapy IR lungs was 51% higher than the IR-0 content. The content in any of the four IR-bitherapy-treated lungs was higher by 64 to 124% than the sums of their corresponding two monotherapies' values and by 46 to 279% more than the IR-0 value. The lowest figure of GSH in either control or IR biotherapy-treated subgroups pertained to the M44N25 lungs, i.e., those treated with the combined two low-dose monotherapies.

Pulmonary Vascular and Ventilatory Data

Pulmonary perfusion pressure values began to increase among the IR-paired lungs starting at the first minute of reperfusion (Fig. 3). The two low-dose IR monotherapies (M44, N25) only slightly reduced the significant (*p < 0.01
pressure increase detected in the IR nontreated (IR-0) lungs. The two high-dose monotherapies (M55, N37) and all four bitherapies were effective in protecting the lungs from injury compared with the controls’ values.

Peak ventilatory pressure (Fig. 4) remained unchanged in all the control and the four bitherapy-treated lungs. The two IR high-dose monotherapy-treated lungs had the pressure in-between the controls’ and the bitherapy-treated lungs’ on the lower level and the IR-0, the IR-M44- or the IR-N25-treated lungs on the higher values. The response patterns of the latter two regimens demonstrated a plateau effect.

**Lung Weight Gain**

There were no significant intra-experimental weight gains in either the control groups, in the four bi-therapy subgroups of lungs or the two IR high-dose monotherapy (M55, N37)-regimen lungs (Fig. 5). In contrast, the two low-dose monotherapy (M44, N25)-treated lungs gained as much weight as did the IR-0 subgroup.

**WDR**

The IR livers’ averaged WDR was $5.3 \pm 0.2$ compared with the averaged WDR of $4 \pm 0.3$ ($p < 0.05$) for the control subgroups of livers, indicating IR-induced liver damage. The IR-M44 and IR-M25 lungs were the only subgroups whose WDRs were similar to that of the IR-0 lung subgroup (Table 2). The rest of the IR-paired and treated lungs, including the ones in the two high-dose monotherapy groups, had WDRs that were similar to those of the controls and they were significantly ($p < 0.01$) lower than the values of the two IR low-dose monotherapy and the IR-0 subgroups (Table 2).

**DISCUSSION**

The hypothesis of there being an additive or even a synergistic protective effect of MN, a ROS scavenger, and NAC, a GSH precursor as well as a ROS scavenger, on reperfusion lung...
damage after liver IR was confirmed in the present study. The postulated ROS-induced alveolocapillary disruption that generates movement of fluid and large molecular weight compounds (e.g., AST) from the vascular compartment into the lung interstitium provided the pathophysiologic basis for the present lung reperfusion injury.\textsuperscript{1} Subsequent demonstration of the dose-specific effectiveness of NAC and of MN in preventing such damage provided the direction for this drug therapy investigation.\textsuperscript{7,8,14} The first important result of this study was the demonstration that the combination of the two lower dose regimens (N25, M44), ineffective when administered individually, prevented ventilatory- and perfusion-related injuries in the IR lung-paired lungs, thus providing evidence of a clear advantage of the combination approach over a single dose monotherapy. This therapeutic effect occurred when any of MN and NAC doses were combined. These drug combinations also yielded an additive XO-reducing activity and augmented the capacity of each of the single doses in replenishing GSH in the IR-paired lungs’ tissues. This latter effect was observed in the controls and in the IRs to a similar degree.

Various double-organ models have been used to assess the direct detrimental effects of hepatic IR in damaging remote organ functions\textsuperscript{1,6} and NAC provided a dose-specific protection of the latter.\textsuperscript{14} The working hypothesis in those studies was that the damaged liver induces remote functional impairment via an oxidative process, which is initiated and maintained in an active state by the high levels of circulating XO, possibly by its generated ROS byproducts.\textsuperscript{1,6–18} Because the effectiveness of NAC in preventing secondary injuries was dose-limited,\textsuperscript{14} as was that of MN,\textsuperscript{7} it was now reasoned that their protective effects on the lung could be augmented if both scavengers were administered in combination.

The various drug combinations comprised two effective monotherapy doses (MN 0.55 mmol and NAC 0.37 mmol) and two ineffective ones (MN 0.44 mmol and NAC 0.25 mmol). The effect of one that could be exerted on the other was investigated by crossmatching them. Thus, the two low doses satisfactorily protected the lungs when given simultaneously because they potentiated each other. Similarly, the combination of M55 with N25 (the M55N25 regimen) which contained the MN dose that had a proven efficacy in isolation and the NAC dose that was ineffective when used alone, was actually more efficient than each of the two regimens alone because of an augmentation effect. Also, when each of the two MN doses was concomitantly administered with NAC 0.37 mmol, the anticipated lung protection (especially in light of the proved M55 and N37 effectiveness) became most apparent by the blockade of XO activity and especially the lung tissue GSH content that increased >twofold compared with the sum of the corresponding monotherapies. There was also only minimal rise in the volumes and the levels of AST that were retrieved in the BAL (as in the IR-N37, IR-M55, or in the IR-M55N25 lungs), which expresses these therapies’ capacity to prevent the overt changes in alveolocapillary permeability (that lead to movements of abnormal amounts of fluids and solutes into the alveoli, as occurs clinically upon the development of acute lung injury). These therapeutic effects coincided with the presently recorded better ventilatory and perfusion parameters and lack of weight gain.

There appear to be no data in the literature to indicate a positive reciprocal effect of the two drugs in reducing primary or secondary lung injury. The reason could be because of their being of dissimilar antioxidant activity. MN is an [\textit{OH}·] scavenger and quenches this and other ROS, thus reducing their injurious activity both extra- and intracellularly.\textsuperscript{3,5} NAC has long been the cornerstone compound used for the investigation of the thiol precursors.\textsuperscript{19} It has been the most frequently investigated glutathione prodrug for the prevention of post-transplant IR effects in the liver\textsuperscript{12} and, because this model could mimic such circumstances, NAC seemed to be the right choice. It was also demonstrated that NAC participates in scavenging XO- and its generated ROS within the circulation and in the lung tissue, besides its enhancing GSH replenishment in the organ.\textsuperscript{14} This study, however, has several limitations in that it neither directly measured [\textit{OH}·] nor other specific ROS nor did it investigate the molecular mechanism by which the drugs’ reciprocal pharmacological lung protection was obtained. ROS were not measured because the direct and precise measurement of free radicals, especially hydroxyls, is difficult to obtain in vivo because of their extremely short half-lives. It has been documented, however, that XO activity faithfully represents ROS’ toxic effects, PMNL recruitment and organ function.\textsuperscript{1,6,16}

Which drug potentiated the other and was the effect merely additive or a synergistic reciprocal one? Did NAC augment MN’s quenching ROS, perhaps via its known oxidant scavenging potentials or, by quenching ROS, did MN enable more NAC to be available for GSH replenishment? The present data failed to provide a conclusive answer to these questions, and more studies will be needed before the issues are put to rest. The bitherapy-additive reduction of XO activity in the BAL and within the tissue could, indeed, be explained by either alternative, because each drug could achieve the same objective, i.e. the re-establishment of the antioxidant dominance.\textsuperscript{6,14,19,20} The fact that XO activity was reduced to even below the control lung activity is not a new finding.\textsuperscript{1} The second marker for antioxidant activity, i.e. the GSH level in the lungs’ tissues, both in the control- and in the IR-added bitherapies, could be explained by MN’s quenching the activity of the XO (and the generated ROS), thus leaving more NAC available to generate more GSH. Indeed, when M44 or M55 were added to either the N25 or N37 regimen, the production of GSH was superior to either NAC single-dose capacity. At the same time, however, potentiation of MN’s quenching effect by NAC would also have led to a similar dose-dependent preservation of GSH because this enzyme would have been consumed to a lesser degree in the presence of a lower circulating stress oxidative activity.\textsuperscript{14} A dose-response protocol aimed at studying either eventualities, as well as the possible synergistic enhancement of the two
drugs (as suggested by the plateau effects of the single doses) is currently under investigation.

The clinical importance of remote organ injury and the possible means for its prevention after organ transplantation lie in the fact that to date none of the organ preservation solutions used by the different transplantation groups has proved to produce consistently optimal results. Thus, livers subjected to prolonged ischemia (during transportation, storage, or grafting) could later induce acute lung reperfusion injury,21 similar to that shown in the current report. Nevertheless, lung dysfunction can also follow limb ischemia22 or the release of a tourniquet23 placed during trauma or severe hemorrhage, and aortic aneurysm repair.9,20,24

In conclusion, the combination of NAC and MN, even at low doses that are not effective when each is given alone, afforded a higher grade of protection against oxidative-induced lung reperfusion injury, when administered as reperfusion process starts. Further studies, however, are still warranted to assess this interaction in other organ and reperfusion models.

ACKNOWLEDGMENTS

We thank Esther Eshkol for editorial assistance.

REFERENCES