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INTESTINAL ISCHEMIA-REPERFUSION INJURY ALTERS PURINERGIC RECEPTOR EXPRESSION IN CLINICALLY RELEVANT EXTRA-INTESTINAL ORGANS

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Abstract

Background—Intestinal ischemia-reperfusion (IIR) injury is known to initiate the systemic inflammatory response syndrome which often progresses to multiple organ failure. We investigated changes in purinoceptor expression in clinically relevant extra-intestinal organs following IIR injury.

Materials and Methods—Anesthetized adult male BalbC mice were randomized to sham laparotomy (control, n=5), or 15 minutes of superior mesenteric artery occlusion. Experimental ischemia was followed by a period of reperfusion [1 minute (n=6) or 1 hour (n=6)]. Mice were then sacrificed and lung, kidney, and intestinal tissues were harvested. Following RNA extraction, purinoceptor mRNA expression for P2Y₂, A₃, P2X₇, A_{2b}, P2Y₄, and P2Y₆, was analyzed using real time RT-PCR.

Results—Significant differences in purinoceptor expression were observed in the lungs and kidneys of mice exposed to IIR injury when compared to controls. Pulmonary P2Y₂ receptor expression was increased in the 1 hour IIR group when compared to control, while pulmonary A₃ receptor expression was incrementally elevated following IIR injury. In the kidney, P2Y₂ receptor expression was increased in the 1 hour IIR group compared to both 1 minute IIR and control, and A₃ receptor expression was decreased in the 1 hour IIR group compared to the 1 minute IIR group. No significant changes were observed in the intestinal purinoceptor profiles.

Conclusion—Purinoceptor expression is altered in the murine lung and kidney, but not intestine following experimental IIR injury. These findings may implicate extracellular nucleotides and purinoceptors as possible mediators of the extra-intestinal organ dysfunction associated with IIR injury.

Keywords

Purinoceptor; Extracellular Nucleotides; ATP; Ischemia-Reperfusion Injury

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DISCLOSURES

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INTRODUCTION

Intestinal ischemia-reperfusion (IIR) injury is a common and devastating problem in hospitalized patients, specifically those in the intensive care setting (1). Regardless of whether IIR injury is the patient's primary pathophysiologic issue, or is secondary to other disease states, progression to the systemic inflammatory response syndrome (SIRS) often ensues (2, 3). Development of acute respiratory distress syndrome (ARDS) and acute renal failure (ARF) are two common sequelae of the malignant progression of SIRS in critically ill patients (4). Many associated pathophysiologic phenomena have been studied, some of which have been implicated as possible mediators of the extra-intestinal organ dysfunction seen during progression of SIRS to multiple organ failure (MOF) (5,6). Areas currently under investigation include the activation of local immune cells (7), alteration of intestinal permeability with subsequent bacterial translocation (8), changes in the local-regional production of inflammatory mediators (9), production of reactive oxygen species (10), changes in coagulation (11), and alteration in the expression of cellular adhesion molecules (12). Despite the extensive efforts in this field of study, the proximal cause of MOF secondary to IIR-induced SIRS has yet to be clearly elucidated. While this devastating progression is common, currently there are no directed therapeutic options available, and clinicians are forced to rely upon supportive measures to treat these patients (13). Although several therapeutic strategies have suggested benefit in the laboratory setting (14–17), none of these interventions have gained clinical significance in the treatment of humans.

Nucleotides have long been recognized as genetic building blocks and repositories for energy storage. Extracellular nucleotide release occurs in a variety of non-excitatory cells in response to mechanical stimulation, noxious stimuli, and ischemia. These ubiquitous molecules can initiate and regulate a myriad of physiologic effects via membrane-bound nucleotide receptors. Purinoceptors are regionally localized, with subtype specificity and activity varying based upon relative agonist profiles and concentrations. Recently, nucleotides have been suggested to play a role in the hypothalamic-pituitary-adrenal axis during times of stress and inflammation (18). Additionally, nucleotides and their receptors modulate acute and chronic inflammatory states (19–22), have been implicated in protection from ischemia-reperfusion injury (23–25), and participate in blood vessel autoregulation (26,27). Furthermore, extracellular nucleotide metabolism may be altered in times of stress (14,28), and purinergic receptor expression plasticity has been shown to occur in response to noxious stimuli (29,30).

Due to their ubiquitous nature and pivotal role in mechanisms of cellular injury and protection, we hypothesized that extracellular nucleotides play a role in extra-intestinal organ dysfunction following IIR injury. Specifically, we sought to characterize purinoceptor expression changes in the intestine and other clinically relevant organs following IIR injury.

MATERIALS AND METHODS

Animal Model

The Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill approved all experimental procedures and protocols. Following a 72-hour period of acclimatization, 17 adult male BalbC mice (wt: 30.0 ± 3.3 grams; Harlan, Indianapolis, IN) were anesthetized via intraperitoneal injection of a ketamine/xylazine/acepromazine solution [Solution ratio 1.0/0.2/0.033; Ketamine 10mg/ml (Ketlar, Monarch Pharmaceuticals, Inc., Bristol, TN), Xylazine 20mg/ml (TranquiVed, Vedco, Inc., St. Joseph, MO), Acepromazine 10mg/ml (Vedco)]. Upon reaching a surgical plane of anesthesia, the animals were randomized to receive either sham laparotomy (control, n=5), or 15 minutes of superior mesenteric artery (SMA) occlusion. Briefly, animals were placed supine on a continuously warmed operating

board and the abdomen was prepped then entered via midline incision. The SMA was reliably identified, and either clamped with an aneurysm clip in the experimental group, or left undisturbed in the control group. The abdomen was closed with suture to prevent heat loss. A 15 minute time period elapsed, the abdomen was reopened, and the aneurysm clip was removed from the SMA in the experimental group. All animals were then resuscitated with approximately 3.3 ml/kg subcutaneous injection of warmed 0.9% NaCl following re-closure of the abdomen. Experimental ischemia was followed by a subsequent period of reperfusion [1 minute (n=6) or 1 hour (n=6)] in the experimental group, or either 1 minute or 1 hour of elapsed time in the control group. The mice were then sacrificed via exsanguination, and lung, kidney, and intestinal (proximal jejunum) tissues were harvested and placed in *RNAlater* (Ambion, Austin, TX) for future analysis.

RNA Analysis

Tissue-Tearor (Biospec Products, Inc., Bartlesville, OK) was used to disrupt the tissue samples, which then underwent RNA extraction using *RNeasy* minikits (Qiagen, Valencia, CA) according to the manufacturer's recommendations. A treatment with RNase-free DNase (Qiagen) was performed on-column according to the manufacturer's protocol. RNA quality was assessed both spectrophotometrically and by denaturing agarose gel electrophoresis. cDNA were prepared using random primers and Moloney's Murine Leukemia Virus Reverse Transcriptase (SuperScript II RNase H-RT, Invitrogen, Carlsbad, CA), as per the manufacturer's instructions. Primer sets were designed to be intron spanning when possible. The NCBI website, Primer3 (Whitehead Institute, Cambridge, MA) and Genosys (Sigma) software applications were used to design the primers (Table 1), which were synthesized by MWG Biotech Inc. (Charlotte, NC). Real-time RT-PCR was performed on a LightCycler Instrument (Roche Diagnostics, Indianapolis, IN), using LightCycler FastStart DNA Master SYBR Green I (Roche), following the manufacturer's instructions. The crossing point (CP) for each sample was determined by LightCycler LCS4 4.00.23 Software (Roche). Each sample was amplified at least in duplicate, for which the CP crossing point standard deviation was <0.5. For each run, a negative control was performed, in which the cDNA template was replaced by water. Melting curves facilitated discrimination between potential primer dimer formation and specific amplified products, and controlled for the homogeneity of a single amplified sequence. Serial dilutions of a control template permitted the establishment of a standard curve. The slope of the linear regression of the CP versus the log cDNA concentration was used to calculate amplification efficiency, as $E = 10^{(1/\text{slope})}$. The relative quantification of gene expression was calculated as a ratio (R), in comparison to a reference gene (cyclophilin A) as previously described (22).

Statistical Analysis

Data is presented as the mean $\pm$ standard deviation (SD). Data was analyzed by ANOVA and Fisher's PLSD tests where appropriate using StatView 5.0.1 Software (SAS Institute Inc., Cary, NC). Significance was defined as $P < 0.05$.

RESULTS

Significant differences in tissue purinergic receptor expression were observed in both the lungs and kidneys of mice exposed to IIR injury when compared to controls. No significant changes were noted in the purinergic receptor expression profiles of the intestinal tissues examined.

P2Y₂

In the lung, P2Y₂ receptor expression was significantly incrementally increased in the 1 hour IIR group when compared to control (1.687 ± 0.556 vs. 1.014 ± 0.259 ; $P < 0.05$). Additionally, significant incremental increases were seen in kidney P2Y₂ receptor expression in the 1 hour

IIR group compared to both 1 minute IIR and control (IIR 1 hr. 1.845 ± 0.816 vs. IIR 1 min. 0.911 ± 0.336 and vs. ctrl. 0.717 ± 0.617 ; $P < 0.05$ for both comparisons). While P2Y₂ receptor expression was detected via real-time RT-PCR in the intestinal tissue, there were no significant changes in expression seen between the various treatment groups or controls. (Figure 1)

A₃

Pulmonary A₃ receptor expression was elevated in an incremental manner following intestinal ischemia-reperfusion injury, with significant differences seen in the 1 hour IIR group compared to both 1 minute IIR and control (IIR 1 hr. 2.093 ± 0.348 vs. IIR 1 min. 1.239 ± 0.750 , and vs. ctrl. 1.042 ± 0.476 ; $P < 0.05$ for both comparisons). A₃ receptor expression in the kidney was significantly decreased in the 1 hour IIR group compared to the 1 minute IIR group (0.496 ± 0.296 vs. 1.436 ± 0.639 ; $P < 0.05$). Intestinal A₃ receptor expression was observed to be present in the tissue samples tested, however no significant changes in receptor expression were noted between the groups examined. (Figure 2)

P2X₇

P2X₇ receptor expression was detected in the lung, kidney, and intestinal tissues examined. There were no significant changes in P2X₇ expression in any of the three tissues. (Figure 3)

A_{2b}

A_{2b} receptor expression was also detected in all of the tissues examined, with approximately twice the relative concentration of receptor mRNA present in the intestine as compared to the kidney and the lung. There was no significant A_{2b} receptor expression changes observed in either of the treatment groups or controls in the lung, kidney, or intestinal tissues examined. (Figure 4)

P2Y₄

Intestinal P2Y₄ receptor expression was found to be present in each of the treatment groups and controls, however no statistical differences were noted between them. P2Y₄ receptor mRNA expression was not detected via real-time RT-PCR analysis in the lung or the kidney tissues of the BalbC mice utilized in these experiments.

P2Y₆

In our experimental conditions, P2Y₆ receptor mRNA expression was not detected in any of the tissues examined via RT-PCR analysis.

DISCUSSION

Intestinal ischemia-reperfusion injury occurring from a wide range of etiologies is a common, and often devastating, problem affecting hospitalized patients. Regardless of its origin, IIR injury often progresses to the systemic inflammatory syndrome, with the subsequent development of multiple organ failure occurring in severe cases (1–3,5). Development of acute respiratory distress syndrome (ARDS) and acute renal failure (ARF) are two common sequelae of the malignant progression of SIRS in critically ill patients (4). Thus, we chose to examine both lung and kidney tissues for purinergic receptor expression changes following IIR injury in order to determine if extracellular nucleotides and their receptors are involved in the extra-intestinal manifestations of this disease process.

Extracellular nucleotides are ubiquitous molecules that initiate and regulate a myriad of physiologic effects via membrane-bound purinoceptors (31). Nucleotide receptors are broadly divided into two categories. P1 Receptors bind adenosine in a concentration-dependent manner,

effecting diverse biological functions via g-protein linked second-messenger systems. There are currently four subtypes of these widely distributed receptors described, with known functions including cellular protection during ischemic states (32), blood vessel autoregulation (33), and regulation of the release of both pro- and anti-inflammatory mediators (34,35). The larger P2 nucleotide-binding group can be further divided into two subfamilies based upon their method of signal transduction. P2X receptors consist of seven known ligand-gated cation channels, whereas P2Y purinoceptor subtypes, of which at least fifteen have been described, act via g-protein coupled IP3 and calcium regulated second messenger systems (36). While some receptor subtypes are highly conserved in structure, function, and localization among species, not all of the described purinoceptors are present in all species (37). Cellular nucleotide release and extracellular metabolism determines the local nucleotide economy, which in turn mediates both paracrine and autocrine functions. The nucleotide economy is altered in times of stress due to cell lysis, altered levels of constitutive release, as well as changes in extracellular metabolism (36).

Nucleotides and purinoceptors have been implicated in many of the associated pathophysiologic phenomena currently being explored as possible mechanisms for the extra-intestinal organ dysfunction seen during progression of SIRS to multiple organ failure. Specifically, nucleotides are known to effect changes in the local-regional production of inflammatory mediators via purine receptor mediated cytokine release (22,38,39). Purinoceptors also serve to mediate changes in the expression of cellular adhesion molecules (40), and have been shown to modulate leukocyte-mediated reactive oxygen species release (41,42). Recently, nucleotides have been suggested to play a role in the hypothalamic-pituitary-adrenal axis during times of stress and inflammation (18), while purinoceptor-modulated acute and chronic inflammatory states have been known to occur for quite some time (19–21). Extracellular nucleotide receptor-mediated tissue injury and protection have been documented following ischemia-reperfusion in the lung (43), kidney (40,44,45), heart (24,46), and brain (47).

Alterations in the expression of purinoceptors have been shown to occur in various tissues in response to noxious stimuli. P2Y₂ receptor expression plasticity has been demonstrated in rat kidneys exposed to hydration and dehydration (30), as well as in submandibular gland cells in a murine model of Sjogren's Syndrome (48). Additionally, our laboratory has demonstrated changes in purinoceptor expression in the rat lung, as well as in the liver and kidney, in response to injurious mechanical ventilation. (29). Taken together, these data suggest a dynamic expression of purinoceptors in response to the cellular environment, both at the site of the stimulus, as well as in distant organs.

P2Y nucleotide receptors are ubiquitously expressed on epithelial cells, with P2Y₂ being the most abundant receptor subtype expressed on the luminal surface of most epithelial cells (49). The pulmonary respiratory epithelium is a site particularly rich in P2Y₂ expression, with its activation linked to mucin production (50), as well as transcellular ion shifts and cellular volume regulation. Specifically, luminal P2Y₂ activation in the respiratory epithelium results in increased Cl⁻ and K⁺ secretion, and inhibition of the electrogenic absorption of Na⁺, producing a net secretion of water into the airways (51–54). Consistent with this cellular mechanism, our laboratory has previously demonstrated that ATP is released into the airways of rats undergoing injurious mechanical ventilation, and this contributes to the development of lung edema (55). In this current study, we demonstrate a significant increase in pulmonary P2Y₂ receptor expression in the one hour IIR group when compared to control. These findings lead us to postulate that perhaps the “wet lungs” commonly seen during the development of ARDS following IIR injury as well as injurious mechanical ventilation, may be due in part to increased expression of P2Y₂, and the subsequent ion and water shifts that accompany changes in this receptor's expression.

P1 adenosine receptors expressed in the respiratory epithelium and on the inflammatory/immune cells in the airways have been implicated in varying, and sometimes contradictory roles ranging from mast cell-mediated bronchoconstriction in chronic inflammatory states, to nucleotide-mediated modulation of both pro- and anti-inflammatory cytokine release (56). Our findings demonstrate pulmonary A₃ receptor expression elevation following IIR injury, with significant differences seen in the 1 hour IIR group compared to both 1 minute IIR and control. The distribution and physiologic effect of A₃ receptors is known to differ among species, and contradictory data within the same species due to differential concentration-dependent P1 receptor activation make deciphering the significance of our findings difficult. Walker *et. al.* demonstrated A₃ receptor mRNA expression elevation in the lung tissue of individuals with chronic inflammation from asthma, although the clinical significance of these findings remains unclear (57). Recently A₃ receptors have been shown to attenuate feline lung reperfusion injury and associated apoptosis via up regulation of the phosphorylated ERK signaling pathway (43). On the contrary, murine data indicate that A₃ receptor activation contributes to airway inflammation via mast cell degranulation and mucus production (58,59). At this time, it is unclear whether elevation of A₃ expression in the lung following IIR injury has protective or deleterious sequelae, and the clinical significance remains to be determined.

We found significant changes in both P2Y₂ and A₃ receptor expression in the kidney following IIR injury. Specifically, significant increases were seen in kidney P2Y₂ receptor expression in the 1 hour IIR group compared to both 1 minute IIR and control. As with the lung, P2Y₂ is abundantly present in the urinary epithelium and participates in transcellular ion shifts and water movement (60). Recent studies have demonstrated that both hypervolemia and ischemia-reperfusion injury induce P2Y₂ expression along the nephron (30,61), thus demonstrating receptor expression plasticity within the organ in response to noxious stimuli, and possibly implicating P2Y₂ as a mechanistic link to the diuresis or water retention that commonly follows such injuries. Additionally, P2Y₂ activation has been implicated in purinoceptor-mediated protection against hypoxia-induced injury in rat proximal tubules (62). Taken together, these data imply that differential P2Y₂ receptor expression in the kidney occurs from both internal and external stimuli, and may serve as an underlying link for potential protective and deleterious renal manifestations seen following P2Y₂ receptor activation.

A₃ expression in the kidney following IIR injury was found to be significantly decreased in the 1 hour IIR group compared to the 1 minute IIR group. While the exact mechanism for downregulation of this purinoceptor remain unclear, decreased levels of A₃ expression in the murine kidney, as evidenced by knockout experiments, confers protection against ischemia and myoglobinuria-induced renal failure (44). One possible explanation may be via decreased apoptotic cell death, as A₃ has been shown to be pro-apoptotic (63). Our data is consistent with a potentially protective physiologic modification in the kidney in response to IIR injury via down regulation of A₃ purinoceptors.

None of the nucleotide receptors examined in this study were observed to have significant expression changes at the site of injury (intestine). Despite the absence of differential expression, our RT-PCR findings document P2Y₂, A₃, P2X₇, A_{2b}, and P2Y₄ but no P2Y₆ receptor expression in the mouse proximal jejunum. Additionally, P2Y₆ mRNA expression was not detected in any of the tissues examined via RT-PCR analysis. While murine P2Y₆ mRNA expression is known to occur in the trachea, neurons, colon, and gallbladder (64–67), and functional studies have suggested that P2Y₆-like receptors reside in the murine kidney and vasculature (68,69), our RT-PCR findings are consistent with the lack of evidence in the literature supporting P2Y₆ localization to the murine kidney, lung, and jejunum.

P2Y₄ receptor mRNA expression was not detected via real-time RT-PCR analysis in the lung or the kidney tissues of the BalbC mice utilized in these experiments. While P2Y₄ receptor

expression has been documented in the murine lung in the past, albeit with far less ferocity than other tissues examined (70), murine P2Y₄ expression in the kidney has not been clearly documented. Recently published data examining noradrenergic and P2 receptor-mediated co-transmission of renal vasoconstriction compared P2Y₄ receptor knockout mice with wild-type littermates, and found no significant differences in renal nerve stimulator induced and exogenously applied ATP- and UTP-mediated pressor responses. The authors concluded that P2Y₄ does not play a role in co-transmission of vasoconstriction (68). The lack of P2Y₄ receptor presence in the murine kidney could possibly explain why no differences were observed between wild type and knockout mice, findings that are in accordance with the results obtained during our RT-PCR analysis.

Regarding our murine model of IIR injury, we chose this system for its ease in carrying out the injurious stimuli, and subsequent tissue collection and analysis. The fifteen minute SMA clamp time was chosen due to its representing the LD₅₀ clamp time for mice used during our prior experiments. Reperfusion times of one minute and one hour were chosen specifically to determine if the receptor expression changes were due primarily to the ischemic insult, as in the case of the relatively short period of reperfusion period of one minute, or from a more significant period of reperfusion injury as evidenced by the one hour reperfusion group. We chose to sacrifice the animals via cardiac puncture and exsanguination while under anesthesia for the simple fact that blood contains a tremendous amount of nucleotides, and purinoceptor-laden cells. Thus, we chose a method for sacrificing the animals that would potentially limit the influx of purinoceptor-laden cells into the tissues during organ harvest.

The notion that changes in purinoceptor mRNA expression occurs in clinically relevant organs following IIR injury raises several questions, not the least of which is whether these receptor changes represent a potential pathophysiologic mechanistic links to the development of MOF. Other possibilities include potential protective strategies employed by the body in order to develop a stance against injury in organs not yet affected by the noxious stimulus. Certainly, the data presented above regarding the specific receptor's physiologic functions point to the potential for both protective and deleterious effects secondary to purinoceptor expression changes. The specific mechanism by which receptor expression occur remains unclear. Obvious sources include the influx and efflux of purinoceptor-laden cells into the tissues following the stimulus, as well as differential parenchymal expression. While determining the source of receptor expression changes will be useful for the potential development of targeted therapies in the future, the actual changes in purinoceptors in various tissues, regardless of their source, are significant and relevant findings due to the tissue effects potentiated by such alterations in receptor quantity. Similarly, the mechanism by which local-regional injury signals changes in distant organs remains unknown.

Currently, other than removing the inciting factor to prevent further IIR injury, clinicians are forced to rely upon supportive measures, as there are no directed therapeutic options available for the treatment of the subsequent organ dysfunction that follows these injuries. While tremendous strides have been taken in understanding the pathophysiologic basis of multiple organ failure following IIR injury, to date, the proximal cause of these events remains unclear. In this article, we present the novel finding that extracellular nucleotide receptor mRNA expression changes in two clinically relevant extra-intestinal organs following IIR injury. These findings may represent a unique proximal mechanistic link that may be modulated in the future to treat critically ill individuals who develop MOF following IIR injury.

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Figure 1.

P2Y₂ receptor gene expression in the lung, kidney, and intestinal tissues of control and intestinal ischemia reperfusion (IIR) experimental mice. Values are means + SD. (* = $P < 0.05$ Control vs. IIR 60 minutes; + = $P < 0.05$ IIR 60 minute vs. IIR 1 minute).

Figure 2. A3 receptor gene expression in the lung, kidney, and intestinal tissues of control and intestinal ischemia reperfusion (IIR) experimental mice. Values are means + SD. (* = $P < 0.05$ Control vs. IIR 60 minutes; + = $P < 0.05$ IIR 60 minutes vs. IIR 1 minute).

Figure 3. P2X₇ gene receptor expression in the lung, kidney, and intestinal tissues of control and intestinal ischemia reperfusion (IIR) experimental mice. Values are means + SD.

Figure 4.

A_{2b} receptor expression in the lung, kidney, and intestinal tissues of control and intestinal ischemia reperfusion (IIR) experimental mice. Values are means + SD.

Table I**OLIGONUCLEOTIDE PRIMERS**

Gene	Forward Primer	Reverse Primer
A_{2b}	cattacagacccccaccaac	aggacccagaggacagcaat
A₃	gtcctgtgtgctgctgatct	gcgcaaacaagaagagaacc
Cyclophilin A	ctgcactgccaagactgaat	tggatgatcttcttctgctg
P2X₇	ccaagccgacgttgaagtat	agagcttggaggtggtgatg
P2Y₂	ctttttgctgtgcccttttc	gaggtcaagtgatcgaagg
P2Y₄	gctctttggctttcccttct	gtccccgtgaacagataga
P2Y₆	cctcacctgcattagcttcc	gctgtcttgggtgatgtggaa

Oligonucleotide primers used to detect gene expression by real-time reverse transcriptase polymerase chain reaction (RT-PCR).