A new target for caffeine in the developing lung: endoplasmic reticulum stress?

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Running head: Caffeine and ER stress in lung development
ABSTRACT

The utility of caffeine to manage apnea of prematurity is widely accepted, however, much controversy surrounds the potential for caffeine to drive post-natal lung maturation in settings of arrested lung development. Many studies have reported pathways relevant to lung injury and lung development are modulated by caffeine in vitro and in vivo, leading to the application of caffeine in experimental animal models of bronchopulmonary dysplasia (BPD). These studies have generated exciting, but at times confusing data. Particularly helpful in understanding the impact of caffeine would be to identify the target molecules or pathways in the developing lung that mediate the effects of caffeine. Here, we critically evaluate a recent report suggesting that endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) are targets of caffeine in a hyperoxia-based rat model of BPD. The authors documented ER stress and engagement of the UPR in the lungs of rats exposed to hyperoxia, where an axis of initiators, transducers, and effectors of the UPR was engaged. The concomitant administration of caffeine to affected rat pups dampened the activity of this axis, leading the authors to conclude that caffeine protects the developing rat lung from injurious stimuli by limiting ER stress and the UPR. The study highlights the need to now directly demonstrate that ER stress and the UPR, and not a plethora of other caffeine-mediated physiological effects, are indeed the relevant targets of caffeine during arrested lung alveolarization.

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The use of caffeine in preterm infants continues to be an exciting and controversial area of neonatal medicine. Caffeine was first used to manage apnea of prematurity in 1977 (3), and commencement of caffeine therapy during the first 10 days of life in very low birth weight preterm infants (weighing 500 to 1250 g at birth) was associated with a reduction in the frequency of bronchopulmonary dysplasia (BPD) and a reduction in the duration of assisted mechanical ventilation (30). While there is general consensus that early caffeine administration is associated with a reduction in the rates of death or BPD and patent ductus arteriosus (21), some concerns remain regarding possible adverse effects, such as increased risk of necrotizing enterocolitis (34). Some investigators have called for further randomized clinical trials to examine early versus late therapy, before caffeine prophylaxis can be universally recommended for infants below 29 weeks of gestational age (16).

Controversy around the use of caffeine in preterm infants is not restricted to the timing of caffeine therapy. Apart from the management of apnea, caffeine has also been suggested to influence post-natal lung maturation, in particular, to stimulate secondary septation and thereby drive lung alveolarization (32). Since preterm infants with “new” BPD exhibit pronounced alveolar simplification as a consequence of stunted secondary septation, the possibility of driving post-natal lung development using a pharmacological intervention that is already established in a neonatal intensive care setting is very exciting indeed. Whether or not caffeine is able to drive lung alveolarization, or protect the developing lung from injurious insults, remains a matter of debate (32).

BPD results from a combination of oxygen toxicity, inflammation, and mechanical stress during respiratory support of affected patients (19). These same injurious stimuli are employed to model BPD in experimental animals (2, 24), including mice (5, 25), rats (27), rabbits (9), pigs (4, 6), lambs (1), and non-human primates (39). At this junction, it is important to recognize the limitation of term animal – particularly rodent – BPD models. The primary utility of caffeine in a neonatal intensive care setting is to reduce apnea, which
reduces the need for mechanical ventilation and oxygen supplementation, by stimulating respiratory efforts. As there is no apnea in mouse pups exposed to hyperoxia, the impact of caffeine on this very important aspect of the clinical consequences of preterm birth is never addressed in animal models (2, 24), even though caffeine also increases the respiratory rate in mouse pups (28). The discussion that follows here is restricted exclusively to the more controversial question of whether, as a side-effect of the use of caffeine to manage apnea, does caffeine administration also influence post-natal lung maturation, in particular, lung alveolarization?

Encouraging data evaluating caffeine as an intervention to drive lung alveolarization in animal models of BPD have been obtained. Studies reporting the use of caffeine and related methylxanthines as pharmacological interventions in experimental animal models of BPD are summarized in the scheme in Fig. 1. Amongst the reports from the “pro-caffeine lobby” are observations that caffeine administration to neonatal rats blunted the lung inflammation that was provoked by hyperoxia (38), and that caffeine administration to preterm rabbits limited the damaging effects of hyperoxia on lung alveolarization, ostensibly also by blunting hyperoxia-provoked inflammation (23). When intra-amniotic bacterial lipopolysaccharide (LPS) was used to mimic chorioamnionitis in pregnant rats, leading to arrested lung alveolarization in offspring, the administration of caffeine (20) or the related methylxanthine theophylline (17, 26), appreciably attenuated the blunted alveolarization, most likely by limiting inflammation. The utility of theophylline to attenuate arrested lung alveolarization in rats caused by hyperoxia exposure has also been demonstrated, also by limiting inflammation (40). Thus, a body of evidence exists highlighting the utility of caffeine to protect or promote lung alveolarization in rat or rabbit models of BPD. However, two other reports in pre-clinical studies either do not support – or actually discourage – the idea that caffeine administration promotes lung alveolarization. One report has documented the deleterious impact of caffeine on lung alveolarization in a hyperoxia-based mouse model of BPD, where caffeine treatment
worsened lung structure (10), while another study reported no impact at all of caffeine administration in the same model (28). Thus, considerable controversy currently exists about whether caffeine has a positive or negative effect – or indeed, no effect – on post-natal lung alveolarization.

A recent report published in the *Journal* (35) provides more ammunition to the “pro-caffeine lobby”. The report of Teng and co-workers (35) identified endoplasmic reticulum (ER) stress (22) as a possible target of caffeine in a hyperoxia-based BPD model in rats. In their study, Teng and co-workers arrested alveolarization in Sprague-Dawley rat pups by exposure to 90% O$_2$ from post-natal day (P)1 to P10, with concomitant daily administration of caffeine (20 mg/kg, intraperitoneal) from P2. Changes in lung structure were assessed by morphometry, where caffeine administration decreased the mean linear intercept (MLI), and increased both the number of secondary septa per high-power field, and the radial alveolar count (RAC). Additionally, lung vascularization was improved, as assessed by the number of rat endothelial cell antigen-stained vessels per high-power field.

Teng and co-workers noted that hyperoxia-exposure caused increased expression of binding immunoglobulin protein (BiP) in the lungs of affected rat pups. As BiP is a master regulator of ER stress, and induction of BiP is regarded as a sign of increased ER stress, this led Teng and co-workers to conclude that hyperoxia drove ER stress. Treatment of newborn rat pups with caffeine concomitant with hyperoxia exposure prevented the hyperoxia-driven expression of BiP. Additionally, the authors provided convincing data that hyperoxia drove the activation of the transducers: protein kinase R (PKR)-like endoplasmic reticulum kinase 1 (PERK; also called eukaryotic translation initiation factor 2-α kinase 3) and inositol-requiring enzyme 1 (IRE1)-α, by phosphorylation; and drove activation of activating transcription factor (ATF)-6, by proteolytic cleavage. These data suggested engagement of the unfolded protein response (UPR). Consistent with the activation of UPR transducers, the expression of two UPR effector molecules: C/EBP homologous protein (CHOP) and X-box binding protein
(XBP)-1 was increased, although immunoblot data supplied to support changes in uXBP1 expression were not convincing, and there is no indication that an outlier test was applied to the densitometry data. The activation of UPR transducers and increased expression of CHOP and XBP1 in response to hyperoxia exposure was attenuated by caffeine treatment concomitant with hyperoxia exposure. In addition to activation of the UPR by hyperoxia, Teng and co-workers also claimed that hyperoxia drove increased COX-2 expression and increased apoptosis (assessed from increased caspase 12 expression), and that both increased COX-2 expression and increased apoptosis were attenuated by caffeine treatment, however, the COX-2 and cleaved caspase 12 immunoblots are difficult to reconcile with the densitometry data provided.

The report of Teng and co-workers is interesting, given the observations that both COX-2 and the ER stress/UPR pathways are activated in the lungs of pre-term infants with BPD (7). In pioneering work from the Bhandari laboratory, COX-2 inhibition, using celecoxib, as well as genetic interference with CHOP expression, attenuated the deleterious impact of hyperoxia on post-natal lung alveolarization in C57BL/6J mice (7). These studies validated a causal role for both COX-2 and CHOP-mediated pathways in hyperoxia-induced arrest of post-natal lung alveolarization. Building on these studies, using a conditional knockout of the ER chaperone 78-kD glucose-regulated protein (GRP78) in lung epithelial cells, the UPR has been proposed as a potential therapeutic target in BPD (15).

The study of Teng and co-workers is limited in that the study reported two separate sets of observations in parallel, but did not functionally connect the two arms of the study. First, the report of Teng and co-workers confirmed the observations of the Bhandari laboratory that COX-2 expression, and ER stress and the UPR, are engaged during hyperoxia-induced arrest of lung alveolarization. In parallel, Teng and co-workers also confirmed the observations of Zhu and co-workers (40), who documented the ability of methylxanthine derivatives to attenuate the impact of hyperoxia on lung alveolarization in
rats. However, no direct functional link was made between hyperoxia-induced arrest of alveolarization and the ability of caffeine to target ER stress and the UPR to attenuate this impact of hyperoxia on alveolarization. To this end, the use of a genetic model, chemical inhibitors, or chemical chaperones such as tauroursodeoxycholic acid (31), in combination with hyperoxia and caffeine administration, would strengthen the suggestions that ER stress and UPR are targeted by caffeine to attenuate the impact of hyperoxia on lung alveolarization.

To the latter point, a vast number of effects of caffeine on the lung have been described, both in vivo and in vitro. In vivo, caffeine has been documented to blunt lung inflammation provoked by hyperoxia in rat pups (38). Furthermore, in vivo, caffeine has been documented to drive alveolar epithelial cell apoptosis (10) and to modulate transforming growth factor (TGF)-β signaling (28) in animals models of BPD. The effects of caffeine on limiting inflammation and other physiological processes in adult animals in response to pro-inflammatory stimuli such as hyperoxia (18), LPS application (29), and ischemia-reperfusion injury (8), have also been described. Further information has been gleaned from in vitro studies, where in epithelial cells, caffeine promotes cell-cycle arrest (14, 36), drives surfactant protein production (11-13), promotes apoptosis (37), and modulates TGF-β signaling (14, 28). In lung fibroblasts and fibroblast-like cell-lines, caffeine impacts peroxynitrite-mediated matrix metalloproteinase production (33), modulates glucocorticoid effects in the lungs (12), and modulates TGF-β signaling (12, 28). Thus, there are many avenues described in lung cells by which caffeine may function to protect lung development from the damaging effects of hyperoxia (summarized in Fig. 2). It remains for Teng and co-workers to demonstrate that the protective effects of caffeine administration in rats is due to caffeine directly targeting ER stress and the UPR, and not via any of the other caffeine-driven physiological effects highlighted above.
The report of Teng and co-workers, which employed rats as an animal model, raised another interesting issue. A screen of the literature revealed that all of the beneficial effects of caffeine that have been noted in rodent models of BPD, have been made in rats, which also holds true for studies in non-human primates and rabbits (Fig. 1, blue routes). In contrast, all caffeine administration studies undertaken in mouse models of BPD have failed to demonstrate any protective effects of caffeine (Fig. 1, red routes): in one study, caffeine worsened the impact of hyperoxia on lung alveolarization in FVB/n mice (10), while in another study, caffeine did not influence the impact of hyperoxia on lung alveolarization in C57BL/6J mice (28). The study of Teng and co-workers in rats was able to document a protective effect of caffeine (by an as-yet-undetermined mechanism) on hyperoxia-induced arrest of lung alveolarization. This protective effect of caffeine and other methylxanthine derivatives has been noted in a variety of rat BPD models (17, 20, 23, 26, 38, 40). Taken together, these reports beg the questions: is there a fundamental difference in the responsiveness of rats versus mice to caffeine, which is relevant to lung alveolarization?

These discordant effects comparing rats to mice might, for example, be attributable to dosing (which is an important consideration in the clinical use of caffeine to manage preterm infants), or to differential expression of caffeine target molecules (such as adenosine receptors and phosphodiesterases) in rats versus mice.

In sum, it is clear that the controversy surrounding the utility of caffeine to manage preterm infants is not restricted to concerns about the timing of the initiation of therapy in the neonatal intensive care unit! In a preclinical setting, many open questions remain concerning whether or not – and how – caffeine may influence post-natal lung maturation in experimental animal models of BPD. Amongst the key questions still to be answered are: (i) What are the in vivo targets and pathways that caffeine modulates to influence aberrant lung development in animal models of BPD? Furthermore, (ii) what underlies the discordant effects of caffeine on lung alveolarization in rat versus mouse models of BPD?
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Fig. 1. Schematic overview of the use of caffeine and related methylxanthines as pharmacological interventions in experimental animal models of lung injury related to BPD. The scheme describes the application of the methylxanthine derivatives theophylline, doxofylline, and caffeine, to mouse, rat, rabbit, and non-human primate models of lung injury induced by including hyperoxia, bacterial lipopolysaccharide, and ischemia/reperfusion. Mouse models generally indicated no beneficial impact of methylxanthine administration (red route), while the non-human ape, rabbit, and rat models generally demonstrated a beneficial impact of caffeine administration (blue route), on distal lung injury. Numbers in parenthesis indicate the relevant citations. (*) Indicates the citation group (8,17,20,26,35,38,40). AEC, alveolar epithelial cell; TGF, transforming growth factor.

Fig. 2. Schematic overview of the impact of caffeine on the physiological properties of lung cells. Specific epithelial and fibroblast cell-types are highlighted in the studies reported in the scheme, along with the relevant physiological process that was explored. Numbers in parenthesis indicate the relevant citations. AEC2, type II alveolar epithelial cell, MMP, matrix metalloproteinase; NF, nuclear factor; p-p53, phospho-p53; TGF, transforming growth factor.
Methylxanthine

Monkey
Rabbit
Rat
Mouse

Injurious stimulus

D i s t a l  l u n g  i n j u r y

Lung function
Mediator expression levels
Mediator localization
Morphology

Improved lung function
Improved alveolarization
Normalized ER stress
Suppressed inflammatory markers
Increased AEC apoptosis
Enhanced inflammatory markers
TGF-β pathway modulation
Arrested alveolarization

(10,28)
(28)
(18)
(23)
(18)
(23)
(23)
(35)
(11,29)
(10,28,29)

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Figure 1
Figure 2

**Fibroblast**
- Caffeine
- Gene regulation involved in airway remodeling
- Peroxynitrite
- MMP-2
- MMP-9
- TGF-β1
- NF-κB

**Epithelial**
- Apoptosis
- Puma
- Synergetic effect
- Cisplatin
- Gene regulation involved in surfactant homeostasis

**Cell cycle arrest**
- SMAD activation

Cell lines:
- IMR-90 (mouse fibroblast)
- A549 (human fibroblast)
- CRL5983
- HTB182
- HTB41
- H441
- MLE-12
- AEC2
- A549