Explorations of the therapeutic potential of influencing metabolism during critical illness

Aslami, H.

Citation for published version (APA):
Pharmacological induction of a hypo-metabolic state with 3-iodothyronamine is not protective in a mouse model of LPS induced lung injury
Abstract

Acute respiratory distress syndrome (ARDS) is a clinical syndrome with a high mortality and morbidity. ARDS is characterized by a hyper-inflammatory response due to a massive influx of neutrophils, associated with mitochondrial damage and subsequent energy failure. 3-iodothyronamine (T1am) is thought to be a thyroid hormone derivate which has opposite effects to thyroid hormone and known to reduce metabolism. We hypothesized that injection of T1am would limit lung injury by reducing metabolism in a mouse model of LPS-induced lung injury. Intratracheal LPS installation induced lung injury, characterized by pulmonary edema and elevated BALF levels of pro-inflammatory cytokines. T1am (50 mg/kg) resulted in cessation of movement shortly after injection. O₂ consumption and CO₂ production was maximally reduced after 2 hours. Six hours after T1am injection, animals were sacrificed and bronchoalveolar lavage fluid (BALF) was obtained. T1am failed to reduce lung injury. In contrast, an increase in BALF levels of TNF-α (7±1.3 vs 10.7±2.5 ng/mL), MIP-2 (3.6±0.6 vs 5.5±1.4 ng/mL) and KC (4.8±1.3 vs. 7.9±1.34 ng/mL, all p<0.05) was observed. Surprisingly, hypometabolism was associated with high plasma T3 levels (mean ± SD) (1.0 ± 0.1 vs. 6.1 ± 2.1 nmol/L) and T4 levels (23.6 ± 4.9 vs. 30.4 ± 6.9 nmol/L) with a decrease in pituitary TSHβ mRNA expression (17.7 ± 6.0 vs. 11.6 ± 1.7 ratio to HPRT, all p<0.05). In conclusion, T1am reduced metabolism in mice with LPS induced ARDS, associated with enhanced lung inflammation and suprafysiological levels of T3. Because of contamination of T1am with trace amounts of T3, no conclusion on the effect of T1am on inflammation can be drawn so far. The finding that metabolism is reduced while T3 levels are high, warrants further investigation.
Introduction

Acute respiratory distress syndrome (ARDS) is a leading cause of mortality and morbidity in the critically ill (1). Excessive production of inflammatory cytokines and chemokines plays a fundamental role in the pathogenesis of ARDS (2), contributing to damage to the epithelial-endothelial membrane, leading to permeability edema (3,4) as well as damage to distant organs. ARDS is further complicated by a failure to live up to an increased energy demand, due to damage to mitochondrial complexes, associated with adverse outcome in lung injury due to pneumonia (5) and sepsis (6). Mechanical ventilation with low tidal volumes is the only effective therapy in ARDS patients (7). However, even with protective ventilation, mortality rates remain high (8), calling for new therapeutic strategies.

Induction of a hypo-metabolic state during excessive inflammation may reduce energy demands and thereby organ injury. Induced hypothermia was found to reduce the hyper-inflammatory response and subsequent lung injury in experimental ARDS models (5,9,10) and was beneficial in patients with severe ARDS (11). Another method which has been applied to reduce metabolism is hydrogen sulfide (H₂S), which was shown to reduce lung injury inflicted by mechanical ventilation (14). However, both methods also have adverse effects (12,13). H₂S has also pro-inflammatory properties and can be toxic (15,16). Since metabolism is also regulated by the hypothalamic-thyroid pathway, specific interference within this pathway may be associated with less detrimental effects.

Thyroxin (T4) is the primary form of thyroid hormone, excreted by the thyroid gland. At the cellular level, T4 is deiodinated to 3,5,3′-triiodothyronine (T3) by iodothyronine deiodinases type 1 (D1) and type 2 (D2). T3 is the active hormone and binds to nuclear thyroid hormone receptor (TR) with a high affinity (17). T3 is inactivated by type 3 deiodinase (D3), one of the deiodinating enzymes (18). Recently, a new thyroid hormone derivate, 3-iodothyronamine (T1am), has been described. T1am, administered in pharmacological amounts, affects metabolism opposite to T3 (19). In mice, T1am caused a reduction in body temperature and bradycardia within minutes after infusion (19). We hypothesize that pharmacological induction of a hypo-metabolic state with T1am reduces LPS-induced lung injury in mouse.

Material and methods

The study was approved by the Animal Care and Use Committee of the Academic Medical Center. Animal procedures were performed in accordance with Institutional Standards for Human Care and Use of Laboratory Animals. Male C57Bl/6 mice (6-8 weeks old, weighing 20-25 grams, Charles River, Maastricht, the Netherlands) were maintained at the animal care facility of the Academic Medical Center according to the institutional guidelines. One day before the experiment, the animals were transferred to the metabolic cage for adaptation.
**Induction of lung injury and T1am treatment**

Under light anaesthesia (2% isoflurane), lung injury was induced by 10 µg/mouse of LPS (E. coli, L4131, Sigma Aldrich, Steinheim, Germany) intratracheally. One hour after LPS, the animals were injected with 50 mg/kg T1am (dissolved in 10% DMSO in 1mL 0.9% NaCl total volume) intraperitoneally. This dosage was chosen based on previous experiments showing maximal metabolic reduction in mice (19;20). Controls received vehicle.

**Experimental protocol and exsanguinations**

O₂ consumption and CO₂ production were measured 4 times hourly by placing a single mouse in a metabolic cage (TSE phenomaster system, Bad Homburg, Germany). Zero calibration was done with 100% nitrogen and high calibration with 21% oxygen according to the manufacturer’s instructions. Data was recorded continuously (TSE phenomaster system software, Bad Homburg, Germany). Six hours after T1am injection, animals were sacrificed by i.p. injection of 7.5 µl/10 gram of body weight of a mix containing 126 mg/kg ketamine, 0.2 mg/kg medetomidine, and 0.5 mg/kg atropine. Bronchoalveolar lavage fluid (BALF) was obtained by flushing the right lung 3 times with 0.5 ml of saline and retrieved immediately. The remaining lobes were used for weighing for calculation of relative lung weight as ratio to body weight or stored in formaldehyde for histopathology. Cell counts were determined using a Coulter cell counter (Beckman Coulter, Fullerton, CA). Differential cell counts were performed on cytospin preparations stained with Giemsa stain in BALF. Parts of the liver as well as the pituitary gland were immediately snap-frozen in liquid nitrogen and stored at -80 for RNA extraction.

**Assays**

Protein levels were determined with Bradford Protein Assay Kit (OZ Biosciences, Marseille, France). Levels of interleukin (IL)–1, IL–6, tumor necrosis factor (TNF)–α, macrophage inflammatory protein (MIP)–2 and keratinocyte-derived chemokine (KC) were measured by ELISA (R&D Systems Inc., Minneapolis, MN) according to the manufacturer’s instructions. Levels of T3 and T4 were measured by in house radioimmunoassay as described (21).

**Histopathology**

After lung wet weight was weighted, the lung lobes were Hematoxylin–Eosin (H&E) stained and analyzed by a pathologist who was blinded for group identity. Interstitial inflammation, endothelialitis, bronchitis and edema were scored on a scale of 0 – 4: 0 for normal lungs, 1 for <25% lung involvement, 2 for 25 – 50% involvement, 3 for 50 – 75% involvement and 4 for >75% lung involvement. Total histology score is the sum score of all parameters.
mRNA extraction and expression

Approximately 10 mg of liver and the whole pituitary gland was used for mRNA extraction with Magna Pure (Roche Molecular Biochemicals, Mannheim, Germany) using the Magna Pure LC mRNA tissue kit. First, strand cDNA synthesis kit with reverse transcriptase polymerase chain reaction (PCR) with oligo d (T) primers (Roche Molecular Biochemicals) were used for cDNA synthesis. We performed Real Time PCR for quantification of hypoxanthine phosphoribosyl transferase (HPRT), D1, D2 D3, TRβ1, TRβ2 and TSHβ using the Lightcycler480 (Roche Molecular Biochemicals, Mannheim, Germany) and the Lightcycler 480 Sybr Green I Master kit (Roche Molecular Biochemicals, Mannheim, Germany as previously described (22). Primers were intron-spanning or genomic DNA contamination was tested using a cDNA synthesis reaction without the addition of Reverse Transcriptase. Samples were corrected for their mRNA content using HPRT as a housekeeping gene because HPRT is suitable to use as a single gene for normalisation in an inflammatory model (23). Samples were individually checked for their PCR-efficiency (24). The median of the efficiency was calculated for each assay, samples that differed more than 0.05 of the efficiency median value were not taken into account. Aberrant PCR-efficiencies occurred randomly and therefore did not bias the results.

Statistical analysis

Statistical analysis was done using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± SEM in the figures. Comparisons between multiple groups were done using analysis of variance followed by Bonferroni post hoc testing. If data were not normally distributed, a Kruskal-Wallis tests was performed with Mann-whitney U test.

Results

The effect of LPS on metabolism and lung injury

After LPS installation, the animals were calm and failed to exhibit exploring behavior in the metabolic cages, while the control animals showed active exploring behavior. In accordance, mice challenged with LPS consumed less O₂ and produced less CO₂ compared to controls (p < 0.05, figure 1). After six hours, O₂ consumption and CO₂ production in LPS-challenged mice had normalized. Intratracheally instilled LPS caused lung injury, characterized by neutrophil influx into the lungs, formation of lung edema and high histopathology score (p < 0.05, figure 2 and figure 3). Also, pulmonary levels of inflammatory cytokines IL-6 and TNF-α, and chemokines MIP2 and KC in BALF were elevated compared to controls (p < 0.05 for all, figure 4). Protein leak into the alveoli was not increased. LPS had no effect on systemic levels of T3
and T4 (figure 5) as well as on liver D1, D3 and TRβ1 mRNA expression (figure 6) or pituitary
D1, D2, TRβ2 and TSHβ mRNA expression (figure 7).

![Graph A](image1)

![Graph B](image2)

Figure 1: 3-iodothyronamine (T1am) reduces metabolism in mice with LPS induced lung injury. Oxygen
(O$_2$) consumption (A) and carbon dioxide (CO$_2$) production (B) during six hours in healthy (saline) and
in mice with LPS induced lung injury treated with vehicle or T1am (n=8 per groups).

The effect of T1am injection on metabolism and on the inflammatory response in LPS induced
lung injury

Shortly after T1am injection, animals in both LPS and control groups stopped moving and
their breathing frequency decreased, with a concomitant drop in O$_2$ consumption and CO$_2$
production (p < 0.05, figure 1). O$_2$ consumption and CO$_2$ production was maximally reduced
two hours after T1am injection and returned gradually to baseline values at six hours. After
approximately 4 hours, animals started moving again.

T1am injection did not reduce lung injury. In contrast, T1am injection resulted in increased
pulmonary levels of TNF-α, KC and MIP2 after six hours compared to LPS challenged mice
injected with vehicle (p < 0.05, figure 2 – 4). Histopathology score tended to be high in the
T1am treated animals. T1am did not provoke an inflammatory response in healthy control
animals.
3-iodothyronamine induced hypo-metabolism is not protective in LPS induced lung injury

Figure 2: 3-iodothyronamine (T1am) had no effect on LPS induced lung injury parameters. Neutrophil influx (A), relative lung weight (B), protein concentrations (C) and histopathology score (D) in healthy controls and in mice with LPS induced lung injury treated with vehicle or T1am. Horizontal line is the mean. BALF: Bronchoalveolar lavage fluids, *: p<0.05, n=8 per groups.

The effect of T1am on thyroid hormone levels
T1am injection was associated with increased amounts of T3 and T4 (p < 0.05, figure 5). Pituitary TSHβ mRNA expression was suppressed in LPS-T1am challenged animals (p < 0.05, figure 7). In saline controls, T1am injection decreased liver TRβ1 and pituitary TRβ2 (p < 0.05, figure 7). D1, D2 and D3 mRNA expression was not affected by T1am (figure 6 and figure 7). Because T3 levels were supraphysiological, we determined the amount of T3 in the administered T1am. We measured 14 nmol/l of T3 in this sample (0.01%).
Figure 3: 3-iodothyronamine (T1am) tended to increase histopathology score in mice with LPS induced lung injury. Representative lung histopathology slides of healthy mice or mice with LPS induced lung injury, treated with vehicle or T1am.

Figure 4: 3-iodothyronamine (T1am) aggravated inflammation in mice with LPS induced lung injury. The levels of interleukin (IL)-1 (A), IL-6 (B), tumor necrosis factor (TNF)-α (C), macrophage inflammatory protein (MIP) -2 (D), keratinocyte chemoattractant (KC) (E) in bronchoalveolar lavage fluids (BALF) in healthy and in mice with LPS induced lung injury treated with vehicle or T1am. Horizontal line is the mean. *: p<0.05, n=8 per groups.
3-iodothyronamine induced hypo-metabolism is not protective in LPS induced lung injury

**Figure 5:** 3-iodothyronamine (T1am) increased thyroid hormones in mice with LPS induced lung injury. Plasma levels of triiodothyronine (T3) and thyroxine (T4) in healthy (saline) mice and in mice with LPS induced lung injury injected with T1am or vehicle. Horizontal line is the mean. *: p<0.05, n=8 per groups.

**Figure 7:** The effect of 3-iodothyronamine (T1am) on thyroid metabolism and regulation in the pituitary. The expression of iodothyronine deiodinases D1 (A), D2 (B), thyroid stimulating hormone (TSH) (C) and thyroid receptor b2 (TRb2) at mRNA level in the liver in healthy (saline) mice and in mice with LPS induced lung injury, injected with T1am or vehicle. The expression of D1, D2, TSH and TRb2 is shown as ration to housekeeping gene HPRT. Horizontal line is the mean. *: p<0.05, n=8 per groups.
Figure 6: The effect of 3-iodothyronamine (T1am) on thyroid metabolism in the liver. The expression of thyroid receptor b2 (TRb2) (A), iodothyronine deiodinases D1 (B) and D3 (C) at mRNA level in the liver in healthy (saline) mice and in mice with LPS induced lung injury, injected with T1am or vehicle. The expression of TRb2, D1 and D3 is shown as ratio to housekeeping gene HPRT. Horizontal line is the mean. *: p<0.05, n=8 per groups.

Discussion

In mice with LPS-induced lung injury, T1am injection caused a reversible metabolic depression which was associated with an exaggerated inflammatory response in the lung. Surprisingly, T1am injection is associated with a marked increase in serum T3 levels, but not in upregulation of T3-responsive genes.

LPS injection resulted in a decrease in metabolism, which presumably was due to less physical activity, as found before (25). Baseline O2 consumption had not fully returned to baseline after 6 hours in the LPS-challenged animals. T1am injection at high doses caused additional hypo-metabolism in spontaneously breathing mice with lung injury, as demonstrated by a decreased O2 consumption and CO2 production. Effects of T1am were transient, returning to baseline levels after 6 hours. Similar physiological changes using the same dose were found after injection of healthy mice with T1am, causing a tremendous drop of 6 to 7°C in body temperature (19) and bradycardia (19;26;27). Our study extends these findings by showing a similar effect in mice with acute lung injury.

Contrary to our hypothesis, limiting energy expenditure using T1am resulted in increased inflammation. This is not in line with previous experiments in models of lung injury in which metabolism was depressed by hypothermia (28) or by using H2S (14). Thereby, we can conclude that inducing a hypo-metabolic state by using T1am is not protective in this ARDS model. We cannot rule out that lower doses of T1am may exert a protective effect during excessive inflammation. However, lower doses may not induce hypo-metabolism (19).

It can be questioned whether detrimental effects of T1am in this study were due to interference with the thyroid axis. Severely ill patients often display profound changes
in thyroid hormone metabolism, termed the non-thyroidal illness syndrome, which is characterized by decreased serum thyroid hormone concentrations. It is hypothesized that decreased serum thyroid hormone levels contribute to an overall down-regulation of metabolism in order to support the immune response and save energy to limit catabolism during critical illness (29;30). Surprisingly, we found that T1am-induced hypo-metabolism was associated with supra-physiological levels of T3, which is difficult to reconcile with decreased O$_2$ consumption and CO$_2$ production. Of note, the method of T3 detection used in this study excludes the possibility of cross-reactivity. Little is known so far about the effects of T1am on thyroid function and we are not aware of the capacity of T1am to induce T3. In order to elucidate the potential mechanisms involved, we measured mRNA expression of deiodinating enzymes. These enzymes are involved in the production and clearance of T3. However, we did not observe alterations in the expression of D1, D2 and D3 in liver and pituitary during this short term experiment, which excludes a role for peripheral conversion. In addition, T1am cannot be converted into T3 (31), suggesting that the excessive amounts of T3 must be derived from the thyroid gland. Alternatively, the T1am compound may have contained amounts of T3. We assayed the compound and indeed, the T1am administered to the mice contained very small amounts of T3. However, because T1am was administered in a pharmacological dose, supraphysiological amounts of T3 were injected.

In the present study, T1am was associated with increased inflammation. In line, hyperthyroid patients have been reported to have elevated levels of circulating inflammatory cytokines (32-34). In particular, high T3 levels was associated with enhanced IL-1, IL-6 and TNF-α production (35). Although the exact mechanism of thyroid hormone induced pro-inflammatory response is unknown, an increase in T3 may play a role in enhancing immune cell function, as was shown for Kupffer cells (36).

In summary, T1am injection reduced metabolism, associated with an aggravated inflammatory response in the lung in mice with LPS-induced lung injury. As the trace amount of T3 in the T1am compound may have resulted in a 10-fold increase in serum T3 levels compared to control mice, no conclusion can be drawn with respect to the effects of T1am on inflammation.
Chapter XII

References

(15) Yen PM. Physiological and molecular basis of thyroid hormone action. Physiol Rev 2001 July;81(3):1097-142.
3-iodothyronamine induced hypo-metabolism is not protective in LPS induced lung injury