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Nicotine and acetylcholine lead to distinct modulation of gene regulation

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Abstract

Background: Sepsis is well known to lead to the activation of multiple pro-inflammatory markers, like MCP-1 (Monocyte chemotactic protein 1), TNF-alpha (Tumor necrosis factor alpha), while the underlying genetic changes still remain poorly studied.

Methods: We used human umbilical vein endothelial cells to test the reactions to nicotine or acetylcholine/pyridostigmine administration in regards to MCP-1 levels, gene regulation and RNR expression.

Results: Pyridostigmine and Acetylcholine (Ach) lead to a significant decrease of MCP-1 levels in TNF-alpha stimulated human umbilical vein endothelial cells, while nicotine had no effect. Interestingly nicotine and acetylcholine lead to different gene expression (nicotine up-regulates epidermal growth factor and down-regulates matrix metalloproteinase-8, while Ach/pyridostigmine up-regulates thioredoxin interacting protein and down-regulates insulin like growth factor 1). Furthermore RNA levels and gene activation were similar after nicotine administration, while changes in RNA levels after Ach/pyridostigmine differed significantly.

Conclusions: Our results suggest that the effects of Ach/pyridostigmine and nicotine are modulated by different underlying pathways, despite their common activation of nicotinic receptors.

Key words: sepsis, gene regulation, gene chips, EGF, TXNIP

Abbreviations

Ach	-	Acetylcholine	MIP	-	Macrophage inflammatory protein
EGF	-	Epidermal growth factor	MMP-8	-	Matrix metalloproteinase
HUVEC	-	Human umbilical vein endothelial cell	TNF-alpha	-	Tumor necrosis factor alpha
IGF-1	-	Insulin like growth factor 1	TXNIP	-	Thioredoxin interacting protein
IL-1	-	Interleukin 1			
IL-6	-	Interleukin 6			
MCP-1	-	Monocyte chemotactic protein 1			

Introduction

It does not need to be emphasized that sepsis is one of the major determinants of morbidity and mortality in intensive care units of industrialized countries around the world. Pathophysiologically sepsis represents a generalized inflammatory response to foreign pathogens. It is well known that this leads to the activation of multiple pro-inflammatory markers, like IL-1, MCP-1, TNF-alpha [1-3]. Therefore, the classic research approach focused on the direct variations on a protein level, while the underlying genetic changes still remain relatively poorly studied. Nevertheless as a result of the recent advances in microbiology and genetics we are becoming more and more aware that sepsis leads to profound regulatory changes of gene activation and expression. It has previously been demonstrated, that sepsis could lead to up-regulation of multiple genes, such as IL-1, Il-6, TNF, MIP or MMP [4].

Besides early antibiotic treatment, effective therapeutic options to treat patients with sepsis only include a very limited number of therapeutic bundles [5]. One of the promising new interventions is the attenuation of the inflammatory response, by activation of the cholinergic anti-inflammatory pathway [6]. We know that activation of this cholinergic anti-inflammatory pathway by vagal stimulation leads to down-regulation of pro-inflammatory markers [7], improves survival in ICU patients sedated with dexmedetomidine [8] and can be utilized to protect against experimental sepsis in multiple studies [6, 9]. This can be accomplished, either directly by injection of nicotine [10], electrical stimulation of the vagal nerve [11] or indirectly by increasing the amount of acetylcholine via cholinesterase inhibition [9]. Furthermore, our group had previously demonstrated that it can also be activated centrally via alpha-2 adrenergic stimulation [6]. Moderate sympatholysis appears to be sufficient to reduce cytokine levels low enough to prevent septic shock and consecutive death, while still allowing an adequate immune response to

clear the infection. To our knowledge there have been no studies conducted so far, linking together the effects of activating the cholinergic anti-inflammatory pathway to changes in gene expression during sepsis.

Here we try to tie together the underlying genetics with the responses we see to nicotine and acetylcholine. We hypothesize that nicotine and acetylcholine stimulation leads to the changes in gene expression we see during sepsis. This could help to explain their distinct effect in modulating the sepsis response via the cholinergic anti-inflammatory pathway.

Materials and methods

Harvesting and cultivation of HUVECs

Human umbilical vein endothelial cells (HUVECs) were isolated as previously described by Jaffe et al. [12]. The umbilical cords were provided by the division of obstetrics and gynecology of the University Hospital of Mannheim Germany and this was in accordance with the ethical standards of the institution.

In brief, after cleaning the umbilical cords with 70% ethanol and canulation of the umbilical vein, we infused phosphate buffered saline (PBS, from XmanufactoX) until all blood was washed out. This was followed by 40min incubation at 37 degrees Celsius with Dispase-II solution to loosen the endothelial layer. After spinning of the cell-suspension at 1500rpm x 5min the supernatant was removed and the remaining cell pallet re-suspended in culture medium at 37 degrees Celsius, 5%CO₂ for 24h till the first change of medium.

HUVECs were grown in culture medium with the addition of 2% fetal calf serum, 1 µg/ml hydrocortison, 0,1 ng/ml hEGF (human endothelial growth factor), 1 ng/ml hbFGF (human basic fibroblast growth factor), 0,4 % ECGS (endothelial cell growth supplement), 5 ng/ml amphotericin B and 50 µg/ml gentamicin. The medium was changed every third day until sufficiently grown. Cells were

washed with PBS and then incubated for 4min at 37 degrees Celsius with Trypsin-EDTA solution until detachment became visible under the microscope. Culture medium was added and the cell suspension centrifuged at 1000 rpm x 5min, before the final suspension of the HUVECs in a 12 well plate.

MCP-1

Concentrations of MCP-1 were measured in human plasma using an enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's instructions (BD Bioscience, San Jose, CA).

Affymetrix gene chips

The effect of nicotine and acetylcholine on transcription levels was screened by using Affymetrix microarrays. Briefly, total RNA from confluent human umbilical vein endothelial cells was isolated and converted to cDNA by reverse transcription. The cDNA was in vitro transcribed into cRNA in the presence of biotinylated dUTP and dCTP. Fragmented biotinylated cRNA was then hybridized to Affymetrix oligonucleotide array U133 Plus 2 (Affymetrix Ltd., High Wycombe, United Kingdom), followed by staining with streptavidin-phycoerythrin. Arrays were scanned and data analysis was performed using GeneChip 3.1 software. When comparing the signals of different arrays, probes must be normalized. Therefore the signal of each probe set was divided by the mean signal of all probe sets from one array (global method of normalization). Gene changes were compared to baseline, untreated conditions. The next 3 steps for determining robust increases (or decreases) of expression were: (i) Increase: Probe sets in the experimental sample with signals below background noise were eliminated from analysis, decrease: Probe sets in the baseline sample with signals below background noise were eliminated from analysis, (ii) only probe sets

with significant increase (decrease) were selected, (iii) only probe sets with an increase (decrease) of the transcript level by > 2-fold (< 0.5-fold) in array experiments were listed.

RT-PCR

Human umbilical vein endothelial cells (HUVEC) were isolated by the method of Jaffe et al. Cells were grown in plates coated with 1% gelatine in endothelial growth medium (Promocell, Heidelberg, Germany) containing 2% FCS as described previously [12].

Statistic and data analysis

Results are presented as either mean \pm SEM or percent change from baseline (100%) as indicated. Statistical significance of $p < 0.05$ was assigned, after t-tests for pairs or non-parametric ranking and oneway ANOVA otherwise. Changes in gene expression were considered significant if the transcript level doubled or halved. Concerning symbolism and higher orders of significance: $p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***.

Results

Pyridostigmine and Acetylcholine lead to a significant decrease in the release of MCP-1 in TNF-alpha stimulated HUVECs, while nicotine has no effect

MCP-1 is significantly upregulated in TNF-alpha (1 ng/ml) stimulated HUVECs compared to baseline (1264 \pm 164pg/ml vs. 1938 \pm 195 pg/ml, $p = 0.03$, $n = 6$). Co-administration of nicotine (1 μ g/ml) and TNF- alpha does not alter the magnitude of MCP-1 levels (1938 \pm 195 pg/ml vs. 2091 \pm 140, $p > 0.05$, $n = 5$). Nevertheless, pyridostigmine (50 μ g/ml) and acetylcholine (0.1 μ g/ml) completely abolish the response to TNF-alpha and restore MCP-1 levels to a level that is insignificantly differ-

ent from baseline (1018.95 ± 217 vs. 1264 ± 164 pg/ml, $p > 0.05$, $n = 4$). This observation gives a first hint that although nicotine and acetylcholine act on the same receptor their effect on MCP-1 activation during inflammation differs significantly.

Nicotine and acetylcholine lead to different gene expression

Next we looked at the underlying pattern of gene expression elicited by nicotine and acetylcholine. Interestingly both molecules lead to distinct up- and down-regulation of different genes (Baseline = 100%), as measured by one affymetrix genechip array looking at a multitude of different genes (see table 1). Nicotine significantly up-regulated endothelium growth factor (EGF, 606%) and down-regulated matrix metalloproteinase-8 (MMP-8, 8 %), while acetylcholine and pyri-

dostigmine did not change either gene regulation. On the flip side, acetylcholine and pyridostigmine significantly up-regulated thioredoxin interacting protein (TXNIP, 348%) and down-regulated insulin like growth factor-1 (IGF-1, 15%), while nicotine did not effect either gene regulation. Interestingly both nicotine and pyridostigmine/acetylcholine had opposing, but only minimal effects on heat shock protein 70 (132% with nicotine and 81% with Ach/pyridostigmine). Those findings illustrate the different effects upon gene regulation of nicotine and Ach/pyridostigmine.

Gene expression differs from RNA production

To elicit if the changes in gene expression translate to changes in RNA levels, we measured RNA levels with real-time PCR of the

Table 1: Gene transcription levels as measured by affymetrix genechip array, showing the different effects upon gene regulation of nicotine and Ach/pyrido. Change shown as % change from baseline (=100%).

	Nicotine vs. control	Ach + pyridostigmine vs. control
EGF	606	100
HSP70	132	081
IGF-1	100	015
MMP-8	008	100
TXNIP	100	348

Table 2: RNA expression as measured by real-time PCR. Change shown as % change from baseline (=100%).

	Nicotine vs. control	Ach + pyridostigmine vs. control
EGF	201	127
HSP70	082	137
IGF-1	090	171
MMP-8	053	035
TXNIP	076	083

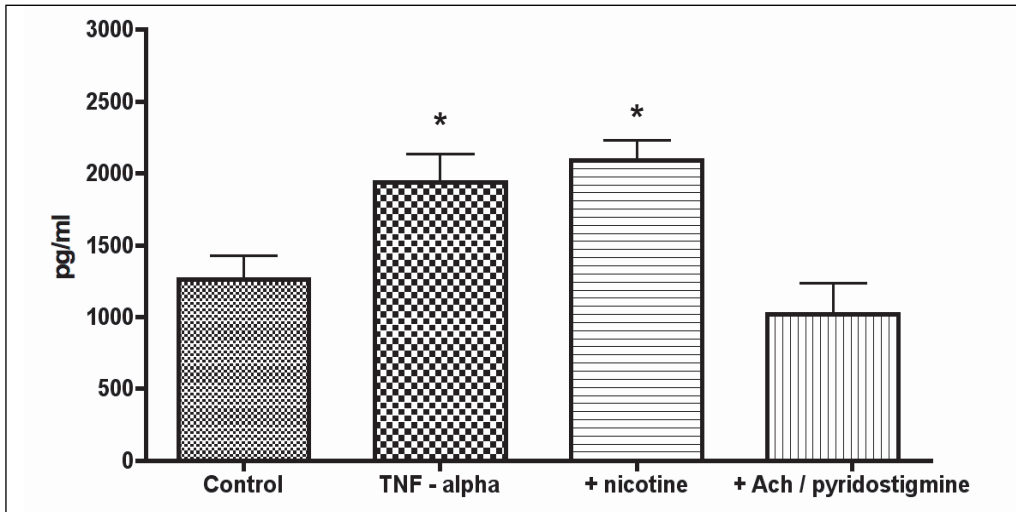


Figure 1: Upregulation of MCP-1 TNF-alpha stimulated HUVECs compared to baseline (1264 ± 164 pg/ml vs. 1938 ± 195 pg/ml, $p=0.03$). This response is unchanged by nicotine co-administration (1938 ± 195 pg/ml vs. 2091 ± 140 , $p>0.05$) but completely abolished with pyridostigmine and acetylcholine administration (1018.95 ± 217 vs. 1264 ± 164 pg/ml, $p>0.05$).

corresponding genes ($n=3$). As expected RNA levels of EGF were significantly higher, while MMP-8 levels were significantly lower after nicotine administration, as predicted by the changes in gene expression. Also TXNIP and HsPA4 levels did not change significantly with nicotine administration. Surprisingly this corresponding behaviour was not true for Ach and pyrido administration. Despite significant changes in gene regulation of IGF-1 and TXNIP, the corresponding RNA levels remained unchanged (171% for IGF-1 and 83% for TXNIP). Furthermore, in contrast to an unchanged gene regulation of MMP-8, the RNA levels of MMP-8 significantly decreased after Ach and pyrido administration (35%). EGF and HsPA4 RNA levels remained unchanged from baseline after Ach and pyrido administration, which is in accordance with the unchanged gene expression (127% for EGF and 137% for HsPA4).

Discussion

Monocyte chemotactic protein 1 (MCP-1) belongs to the chemokine family and recruits monocytes, memory T-cells and dendritic cells [13, 14]. In this study we show that inflammation leads to a marked upregulation of MCP-1 levels that can be reversed by incubation with acetylcholine and pyridostigmine, but not with nicotine. We therefore conclude that it is not the activation of the nicotine receptor that leads to protection against sepsis but that there is a second underlying pathway involved, that is independent from activation of the nicotinic acetylcholine receptor.

It has been shown previously that both nicotine and acetylcholine can activate the cholinergic anti-inflammatory pathway by increasing vagal tone [6]. This activation leads to a downregulation of pro-inflammatory cytokines and has also been shown to improve survival in experimental sepsis [6]. As both drugs have similar effects on the vagal nerve it is unlikely that this is the cause of the different effect on MCP-1 levels.

In this light the findings from the affymetrix gene array are very interesting. We are able to show, that acetylcholine and nicotine lead to a different activation of genes and are therefore activating different secondary pathways besides their established primary function. Nicotine activates EGF and downregulates MMP-8, therefore interacting closely with vessel vasodilation [15] and inflammation control [16]. While acetylcholine/pyridostigmine activates TXNIP and downregulates IGF-1, linking its action closely to muscle metabolism and metabolism (IGF-1), as well as antioxidant pathways (TXNIP).

There are several shortcomings of our study, which include the lack of confirmation of the results on a protein level via Western-blot or ELISA. Therefore we cannot account for posttranslational changes or alternative splicing, that might alter the effects on the body.

Our results demonstrate that the effects of acetylcholine/pyridostigmine and nicotine are modulated by different underlying pathways, despite their common activation of nicotinic receptors.

Speculations

To our knowledge this is the first study looking at the expression of different genes in response to nicotine and Ach/pyridostigmine stimulation. Our results show that those mediators potentially modulate different pathways, even though they all act via nicotinic receptors. This should prompt further research to elicit those underlying pathways which might explain the protective effect of nicotinic receptor activation and thereby activation of the cholinergic anti-inflammatory pathway in sepsis.

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References

1. Pruitt JH, Copeland EM, 3rd, Moldawer LL. Interleukin-1 and interleukin-1 antagonism in sepsis, systemic inflammatory response syndrome, and septic shock. *Shock* 1995; 3: 235-251
2. Kurt AN, Aygun AD, Godekmerdan A, Kurt A, Dogan Y, Yilmaz E. Serum IL-1beta, IL-6, IL-8, and TNF-alpha levels in early diagnosis and management of neonatal sepsis. *Mediators Inflamm* 2007; 31397
3. Ramnath RD, Ng SW, Guglielmotti A, Bhatta M. Role of MCP-1 in endotoxemia and sepsis. *Int Immunopharmacol* 2008; 8: 810-818
4. Suzuki T, Hashimoto S, Toyoda N, Nagai S, Yamazaki N, Dong HY, Sakai J, Yamashita T, Nukiwa T, Matsushima K. Comprehensive gene expression profile of LPS-stimulated human monocytes by SAGE. *Blood* 2000; 96: 2584-2591
5. Brunkhorst FM, Reinhart K. Sepsis therapy: Present guidelines and their application. *Chirurg* 2008
6. Hofer S, Steppan J, Wagner T, Funke B, Lichtenstern C, Martin E, Graf BM, Bierhaus A, Weigand MA. Central sympatholytics prolong survival in experimental sepsis. *Crit Care* 2009; 13: R11
7. Kim MH, Hahn TH. The effect of clonidine pretreatment on the perioperative proinflammatory cytokines, cortisol, and ACTH responses in patients undergoing total abdominal hysterectomy. *Anesth Analg* 2000; 90: 1441-1444
8. Spies CD, Dubisz N, Neumann T, Blum S, Muller C, Rommelspacher H, Brummer G, Specht M, Sanft C, Hannemann L, Striebel HW, Schaffartzik W. Therapy of alcohol withdrawal syndrome in intensive care unit patients following trauma: results of a prospective, randomized trial. *Crit Care Med* 1996; 24: 414-422
9. Hofer S, Eisenbach C, Lukic IK, Schneider L, Bode K, Brueckmann M, Mautner S, Wente MN, Encke J, Werner J, Dalpke AH, Stremmel W, Nawroth PP, Martin E, Krammer PH, Bierhaus A, Weigand MA. Pharmacologic cholinesterase inhibition improves survival in experimental sepsis. *Crit Care Med* 2008; 36: 404-408

10. Liliensiek B, Weigand MA, Bierhaus A, Nicklas W, Kasper M, Hofer S, Plachky J, Grone HJ, Kurschus FC, Schmidt AM, Yan SD, Martin E, Schleicher E, Stern DM, Hammerling GG, Nawroth PP, Arnold B. Receptor for advanced glycation end products (RAGE) regulates sepsis but not the adaptive immune response. *J Clin Invest* 2004; 113: 1641-1650
11. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 2000; 405: 458-462
12. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 1973; 52: 2745-2756
13. Carr MW, Roth SJ, Luther E, Rose SS, Springer TA. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc Natl Acad Sci U S A* 1994; 91: 3652-3656
14. Xu LL, Warren MK, Rose WL, Gong W, Wang JM. Human recombinant monocyte chemotactic protein and other C-C chemokines bind and induce directional migration of dendritic cells in vitro. *J Leukoc Biol* 1996; 60: 365-371
15. Zhou Y, Brigstock D, Besner GE. Heparin-binding EGF-like growth factor is a potent dilator of terminal mesenteric arterioles. *Microvasc Res* 2009; 78: 78-85
16. Gutierrez-Fernandez A, Inada M, Balbin M, Fueyo A, Pitiot AS, Astudillo A, Hirose K, Hirata M, Shapiro SD, Noel A, Werb Z, Krane SM, Lopez-Otin C, Puente XS. Increased inflammation delays wound healing in mice deficient in collagenase-2 (MMP-8). *FASEB J* 2007; 21: 2580-2591

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