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Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin

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Vertebrates achieve internal homeostasis during infection or injury by balancing the activities of proinflammatory and anti-inflammatory pathways. Endotoxin (lipopolysaccharide), produced by all gram-negative bacteria, activates macrophages to release cytokines that are potentially lethal[1–4]. The central nervous system regulates systemic inflammatory responses to endotoxin through humoral mechanisms[5–8]. Activation of afferent vagus nerve fibres by endotoxin or cytokines stimulates hypothalamic–pituitary–adrenal anti-inflammatory responses[9–11]. However, comparatively little is known about the role of efferent vagus nerve signalling in modulating inflammation. Here, we describe a previously unrecognized, parasympathetic anti-inflammatory pathway by which the brain modulates systemic inflammatory responses to endotoxin. Acetylcholine, the principle vagal neurotransmitter, significantly attenuated the release of cytokines (tumour necrosis factor (TNF), interleukin (IL)-1β, IL-6 and IL-18), but not the anti-inflammatory cytokine IL-10, in lipopolysaccharide-stimulated human macrophage cultures. Direct electrical stimulation of the peripheral vagus nerve *in vivo* during lethal endotoxaemia in rats inhibited TNF synthesis in liver, attenuated peak serum TNF amounts, and prevented the development of shock.

Vagus nerve signalling is a critical component of the afferent loop that modulates the adrenocorticotropin and fever responses to systemic endotoxaemia and cytokinaemia[12–15]. Efferent vagus nerve signalling may facilitate lymphocyte release from thymus through a nicotinic acetylcholine receptor response[16]. Clinical studies indicate that nicotine administration can be effective for treating some cases of inflammatory bowel disease[17,18], and that proinflammatory cytokines are significantly decreased in the colonic mucosa of smokers with inflammatory bowel disease[19]. Accordingly, we reasoned that the cholinergic parasympathetic nervous system may modulate the systemic inflammatory response.

We established primary human macrophage cultures by incubating human peripheral blood mononuclear cells in the presence of macrophage colony stimulating factor (MCSF). Acetylcholine (ACh) inhibited TNF release dose-dependently in macrophage cultures conditioned by exposure to lipopolysaccharide (LPS) for 4 h (Fig. 1a). We observed a comparable inhibition of TNF release by ACh from macrophages exposed to LPS for 20 h (data not shown), indicating that ACh did not merely delay the onset of the TNF response. We also treated macrophage cultures with carbachol, a cholinergic agonist chemically distinct from ACh, and observed

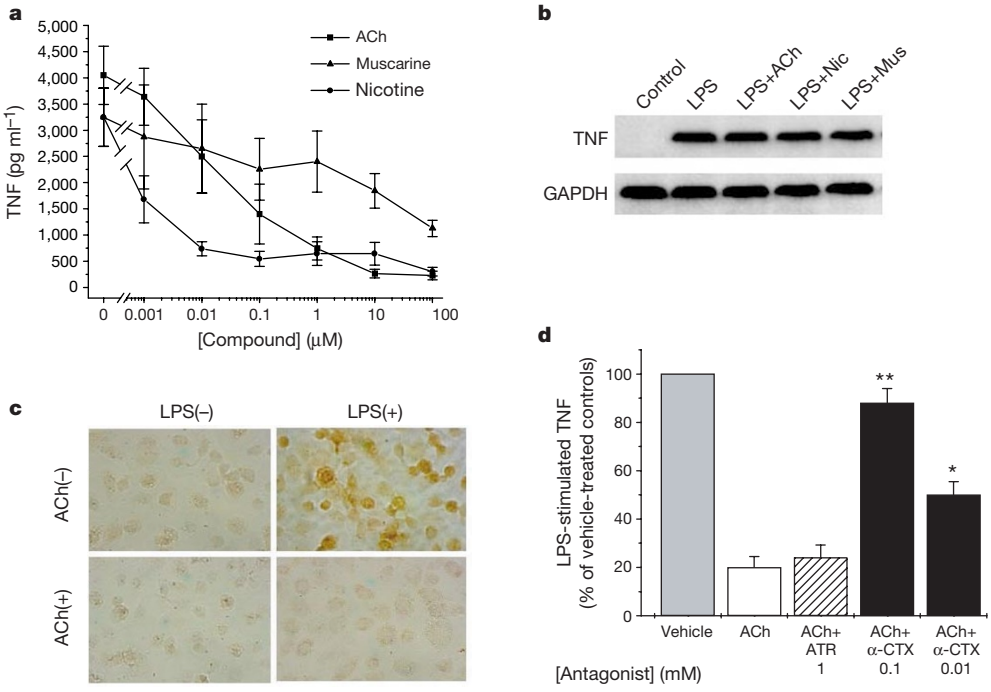

Figure 1 Cholinergic agonists inhibit LPS-induced TNF synthesis in human macrophage cultures through a post-transcriptional mechanism. **a**, Dose-dependent inhibition of TNF release by ACh (squares), muscarine (triangles) and nicotine (circles) after stimulation with LPS for 4 h. Data are mean ± s.e.m; $n = 9$. **b**, ACh (100 μM), muscarine (100 μM, Mus) and nicotine (100 μM, Nic) do not reduce amount of LPS-stimulated (2 h)TNF mRNA in macrophages. **c**, Immunostaining with anti-TNF antibodies reveals a substantial decrease in LPS-induced (2 h) TNF immunoreactivity in ACh-treated (100 μM) human macrophages. **d**, α-Conotoxin (α-CTX), but not atropine (ATR), restores the LPS-stimulated release of TNF in cultures treated with ACh (10 μM). Neither atropine nor α-conotoxin altered TNF production in vehicle-treated cultures (not shown). Data shown are mean ± s.e.m. of three separate experiments. Asterisk, $P < 0.05$ versus ACh; double asterisk, $P < 0.005$ versus ACh.

inhibition of TNF release (data not shown). We investigated the molecular mechanism of TNF inhibition by measuring TNF messenger RNA amounts in an RNase protection assay. The amount of TNF mRNA in ACh-treated, LPS-stimulated macrophages did not decrease compared with vehicle-treated, LPS-stimulated macrophages, even when ACh was added in concentrations that inhibited TNF protein release (Fig. 1b). This indicates that ACh suppresses TNF through a post-transcriptional mechanism. To determine whether ACh inhibited TNF synthesis or release, we labelled cell-associated TNF in human macrophages using monoclonal anti-TNF antibodies. ACh significantly attenuated LPS-stimulated TNF immunoreactivity in macrophages (Fig. 1c), indicating that the inhibitory effect of ACh on human macrophage TNF production occurs through the post-transcriptional suppression of TNF protein synthesis.

Peripheral blood mononuclear cells express nicotinic and muscarinic ACh receptors[20–22]. To define pharmacologically the type of macrophage cholinergic receptors involved in modulating the TNF response, we measured TNF in macrophage culture medium exposed to LPS for 4 h in the presence of either muscarinic or nicotinic agonists of ACh receptors (Fig. 1a). Nicotine significantly inhibited TNF release in a dose-dependent manner. The effective concentration of nicotine that inhibited 50% of the TNF response (EC_{50}) was estimated to be 8.3 ± 7.1 nM ($n = 9$). This compared favourably with the EC_{50} for ACh-mediated inhibition of TNF (ACh $EC_{50} = 20.2 \pm 8.7$ nM, $n = 9$). Muscarine also significantly inhibited TNF release, although it was much less effective than either ACh or nicotine (muscarine $EC_{50} = 42.4 \pm 18.6$ μM, $n = 9$; $P < 0.01$ versus nicotine or ACh). As these results did not establish whether ACh inhibited TNF primarily through the activity of nicotinic or muscarinic ACh receptors, we added the specific muscarinic antagonist, atropine, to LPS-stimulated macrophage cultures co-treated with ACh (Fig. 1d). Addition of atropine, even in concentrations as high as 1 mM, failed to restore TNF release in ACh-treated macrophage cultures. We next addressed whether the nicotinic ACh receptor activity that mediated inhibition of TNF was sensitive to α-bungarotoxin. Addition of α-conotoxin to ACh-treated LPS-stimulated macrophage cultures significantly and dose-dependently reversed the inhibitory effect of ACh (Fig. 1d). These data indicate that the inhibitory effect of ACh on the LPS-induced TNF response in human macrophage cultures is mediated primarily by α-bungarotoxin-sensitive, nicotinic ACh receptors. The amount of ACh in mammalian tissues can reach the millimolar range[23], so it is possible that both the nicotinic and muscarinic macrophage ACh receptors participate in the inhibition of macrophage TNF synthesis *in vivo*.

To assess the specificity of the cytokine response to ACh, we measured the release of other macrophage-derived cytokines in LPS-stimulated, ACh-treated macrophage cultures. ACh dose-dependently inhibited the release of other LPS-inducible cytokines (IL-1β, IL-6 and IL-18), but failed to prevent the constitutive release of the anti-inflammatory cytokine IL-10 (Fig. 2). Staining with tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), and Trypan blue exclusion of macrophage cultures treated with LPS and ACh, indicated that specific LPS-inducible cytokine inhibition was not because of cytotoxicity (data not shown). The molecular mechanism of ACh inhibition of IL-1β and IL-6 was investigated further by measuring gene-specific mRNA amounts with biotin-labelled capture oligonucleotide probes in a microplate assay (Quantikine mRNA, R&D Systems). Stimulation of human macrophage cultures with LPS for 2 h significantly increased the mRNA amounts of IL-1β compared with vehicle-treated controls (vehicle-treated IL-1β mRNA = 120 ± 54 attomole (amol) ml^{-1} versus LPS-stimulated IL-1β mRNA = 1974 ± 179 (amol) attomole ml^{-1}; $n = 3$; $P < 0.01$). Addition of ACh in concentrations that inhibited IL-1β protein release (100 nM) did

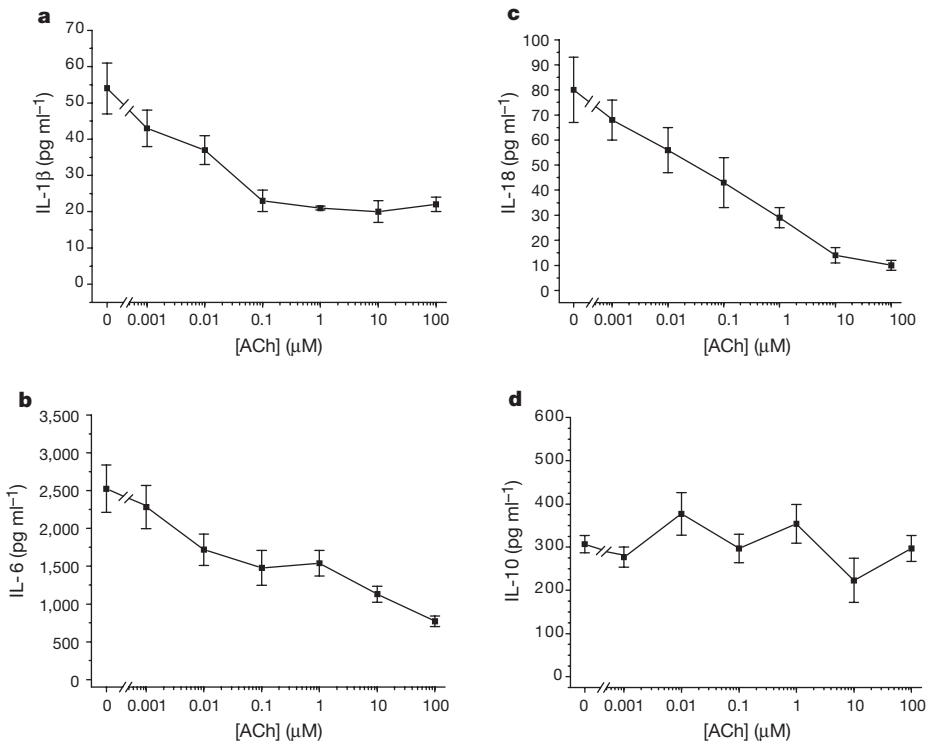

Figure 2 ACh specifically inhibits release of pro-inflammatory cytokines by human macrophages, but does not suppress release of the anti-inflammatory cytokine, IL-10. **a–d**, LPS-stimulated human macrophage cultures were incubated with ACh at the indicated concentrations in the presence of pyridostigmine bromide (1 mM) and LPS (100 ng ml^{-1}). IL-1β (**a**), IL-6 (**b**), IL-18 (**c**) and IL-10 (**d**) amounts were measured in conditioned media (20 h) using commercially available ELISA kits. Data are mean ± s.e.m. from four separate experiments.

not significantly alter macrophage IL-1β mRNA amounts (ACh-treated LPS-stimulated IL-1β mRNA = 2128 ± 65 amol ml^{-1}; $n = 3$). Similarly, LPS-stimulated IL-6 mRNA amounts in macrophages were not significantly altered by ACh concentrations that inhibited IL-6 protein (LPS-stimulated IL-6 mRNA = 1716 ± 157 amol ml^{-1} versus ACh-treated LPS-stimulated IL-6 mRNA = 1872 ± 91 amol ml^{-1}; $n = 3$). These observations indicate that ACh post-transcriptionally inhibits the LPS-stimulated release of TNF, IL-1β and IL-6 in macrophages.

To determine whether direct stimulation of efferent vagus nerve activity might suppress the systemic inflammatory response to endotoxin, we subjected adult male Lewis rats to bilateral cervical vagotomy, or a comparable sham surgical procedure in which the vagus nerves were isolated but not transected. Efferent vagus nerve activity was stimulated in vagotomized rats by application of constant voltage pulses to the distal end of the divided vagus nerve 10 min before and again 10 min after the administration of a lethal LPS dose (15 mg kg^{-1}, intravenous). Electrical stimulation of the efferent vagus nerve significantly decreased the amounts of TNF in the serum. By contrast, vagotomy without electrical stimulation significantly increased peak serum TNF amounts compared with sham-operated controls ($P < 0.05$) (Fig. 3a). We next measured TNF in liver homogenates, because liver is a principal source of serum TNF during endotoxaemia[24,25]. Electrical stimulation of the distal vagus nerve decreased hepatic LPS-stimulated TNF synthesis compared with sham-operated controls. Vagotomy without stimulation was associated with increased TNF synthesis in liver (Fig. 3b). These data directly implicate efferent vagus nerve signalling in the regulation of TNF production *in vivo*.

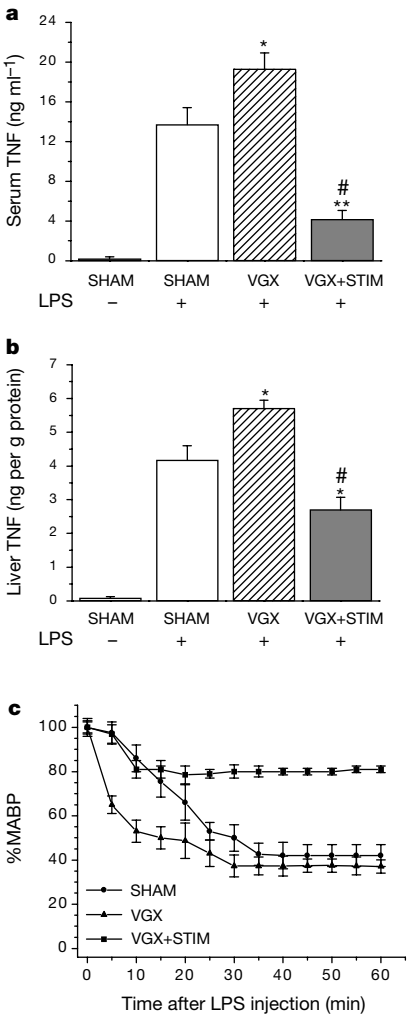

Figure 3 Vagus nerve stimulation attenuates the LPS-induced serum TNF response, hepatic TNF response, and development of endotoxic shock. Rats were subjected to either sham surgery (SHAM, white bars (**a, b**) or circles (**c**), $n = 7$), bilateral cervical vagotomy (VGX, striped bars (**a**, **b**) or triangles (**c**), $n = 7$) or vagotomy and electrical stimulation (VGX + STIM, shaded bars (**a**, **b**) or squares (**c**), $n = 7$). **a**, Serum TNF response. **b**, hepatic TNF response. **c**, Development of endotoxic shock. Mean arterial blood pressure data are normalized to MABP at time = 0. Sham-surgery, vagotomy and electrical stimulation with vagotomy did not significantly affect MABP in vehicle-treated controls (not shown). Data are mean ± s.e.m. Asterisk, $P < 0.05$; double asterisk, $P < 0.005$ versus SHAM + LPS; hash, $P < 0.05$ versus VGX + LPS.

It was possible that electrical stimulation of the vagus nerve induced the release of humoral anti-inflammatory hormones or cytokines that inhibit TNF production. Measurement of corticosterone and IL-10 in sham-operated controls (Table 1) revealed that endotoxaemia was associated with increases in the amount of corticosterone and IL-10. In agreement with previous studies, vagotomy significantly reduced the amount of corticosterone, in part because it eliminated the afferent vagus nerve signals to the brain that are required for a subsequent activation of the hypothalamic–pituitary–adrenal signalling pathway[12,13]. This decreased corticosteroid response probably contributed to the increased amounts of TNF observed in the serum and liver of vagotomized animals (Fig. 3) because corticosteroids normally downregulate TNF production[10,11]. Direct electrical stimulation of the peripheral vagus nerve did not stimulate an increase in either the corticosteroid or the IL-10 responses. Thus, suppressed TNF synthesis in the serum and liver after vagus nerve stimulation could not be attributed to the activity of these humoral anti-inflammatory mediators.

Peripheral vagus nerve stimulation significantly attenuated the development of LPS-induced hypotension (shock) in rats exposed to lethal doses of endotoxin (Fig. 3c). This was expected, as TNF is a principle early mediator of acute endotoxin-induced shock[1,26]. Vagotomy without stimulation significantly shortened the time to development of shock compared with sham-operated controls (time to 50% drop in mean arterial blood pressure: sham = 30 ± 3 min versus vagotomy = 15 ± 2 min; $P < 0.05$). This amplified development of shock following vagotomy corresponds to the decreased corticosteroid response and the increased TNF response. ACh is a vasodilator that mediates nitric oxide-dependent relaxation of resistance in blood vessels causing a decrease in blood pressure. We wished to exclude the possibility that stimulation of the efferent vagus might have mediated a paradoxical hypertensive response. Hypertension was not observed following vagus nerve stimulation of controls given saline instead of endotoxin (not shown), indicating that protection against endotoxic shock by vagus nerve stimulation is specific. These observations indicate that stimulation of efferent vagus nerve activity downregulates systemic TNF production and the development of shock during lethal endotoxaemia.

The present results show that differentiated human macrophage cultures are extremely sensitive to ACh and nicotine. Previous reports of cholinergic receptor activity in human peripheral blood mononuclear cells that were not differentiated into macrophages[21,27,28]

Table 1 Effects of vagotomy and vagus nerve stimulation on serum IL-10 and corticosteroid amounts during lethal endotoxemia

Group of animals	IL-10 (ng ml^{-1})	Corticosterone (ng ml^{-1})
SHAM + vehicle	n.d.	160 ± 20
SHAM + LPS	8 ± 0.3	850 ± 50
Vagotomy + LPS	9 ± 0.4	570 ± 34*
Vagotomy + LPS + Stimulation	9 ± 0.5	560 ± 43*

Animals were subjected to either sham surgery (SHAM), vagotomy (VGX), or electrical stimulation with vagotomy (VGX+STIM) 30 min before systemic administration of LPS (15 mg kg^{-1}). Blood samples were collected 1 h after administration of LPS or vehicle. Serum corticosterone was measured by radioimmunoassay (ICN Biomedicals, Costa Mesa, CA) and IL-10 was determined by ELISA (BioSource International, Camarillo, CA). All assays were performed in triplicate. Data shown are mean ± s.e.m., $n = 7$ animals per group. Asterisk, $P < 0.05$ versus SHAM + LPS; n.d., not detectable.

implied that maximal cholinergic responses required micromolar concentrations of cholinergic agonists. Our own studies of undifferentiated human peripheral blood mononuclear cells confirmed that significantly higher concentrations of ACh are required to suppress cytokine synthesis (ACh EC_{50} for inhibiting TNF in peripheral blood mononuclear cells = 0.8 ± 0.2 mM, $n = 3$). The pharmacological results now implicate an α-bungarotoxin-sensitive, nicotinic ACh receptor activity that can modulate the macrophage cytokine response. This type of cholinergic receptor activity is similar to that previously described in peripheral blood mononuclear cells[21], except that macrophages are significantly more sensitive to cholinergic agonists than peripheral blood mononuclear cells.

The neural–immune interaction described here—which we call the 'cholinergic anti-inflammatory pathway'—can directly modulate the systemic response to pathogenic invasion. The observation that parasympathetic nervous system activity influences circulating TNF amounts and the shock response to endotoxaemia has widespread implications, because it is a previously unrecognized, direct and rapid endogenous mechanism that can suppress the lethal effects of biological toxins. The cholinergic anti-inflammatory pathway has much shorter response times than the humoral anti-inflammatory pathways. Moreover, activation of parasympathetic efferents during systemic stress, or the 'fight or flight' response, confers an additional protective advantage to the host by restraining the magnitude of a potentially lethal peripheral immune response. □

Methods

Human macrophage cultures

Buffy coats were collected from the blood of healthy individual donors to the Long Island Blood Bank Services (Melville, New York). Primary blood mononuclear cells were isolated by density-gradient centrifugation through Ficol/Hypaque (Pharmacia), suspended (8×10^6 cells ml^{-1}) in RPMI 1640 medium with 10% heat inactivated human serum (Gemini Bio-Products), and seeded in flasks. After incubation for 2 h at 37 °C, adherent cells were detached with 10 mM EDTA, resuspended (10^6 cells ml^{-1}) in medium supplemented with human MCSF (Sigma; 2 ng ml^{-1}), and seeded onto 24-well tissue culture plates (10^6 cells per well). Cells were allowed to differentiate for seven days in the presence of MCSF.

On day seven, fresh medium without MCSF was added, and experiments performed as indicated. Human macrophage cultures were exposed to ACh chloride (Sigma), muscarine or nicotine where indicated. All experiments using ACh included the ACh esterase inhibitor pyridostigmine bromide (1 mM, Sigma). Five minutes after the addition of the cholinergic agents, LPS was added to the cultures at a final concentration of 100 ng ml^{-1}. Supernatants were collected 4 or 20 h after the addition of LPS and prepared for cytokine ELISA in accordance with the manufacturers' instructions. ELISA kits were obtained from R&D Systems (TNF, IL-1β, IL-6 and IL-10) and Medical & Biological Laboratories (IL-18). For experiments using atropine (Sigma) or α-conotoxin (Oncogene), the cholinergic anatagonists were added to the macrophage cultures 5 min before ACh (10 μM) and LPS.

RNase protection assay

Macrophages were incubated with 100 μM ACh, muscarine (Mus) or nicotine (Nic) for 5 min followed by 2 h exposure to LPS. Control wells had medium alone. The RNase protection assay was conducted using a kit obtained from PharMingen. The anti-sense RNA probe set (hck-3) was labelled with [α-^{32}P]UTP (800 Ci mmol^{-1}, Amersham) using T7 RNA polymerase. Molecular weight markers were prepared by using pBR-322 plasmid DNA digested with *Msp*I (New England Bio Labs) and end-labelled using [α-^{32}P]dCTP (800 Ci mmol^{-1}, Amersham) with Klenow enzyme (Strategene). Expression of the GAPDH gene product was measured to control for mRNA loading. Total RNA was isolated from cultured cells by using TRIzol reagent (GIBCO BRL) following the manufacturer's instructions.

TNF immunohistochemistry

Human macrophages were differentiated as described above, and grown on glass chamber slides. Cells were exposed to ACh (100 μM) in the presence of pyridostigmine bromide (1 mM), 5 min before LPS treatment. Two hours later the cells were fixed in buffered 10% formalin and subjected to immunocytochemical analysis. Slides were incubated in a blocking solution (1% BSA, 5% normal goat serum, 0.3% Triton X-100 in PBS) for 1 h at room temperature and then incubated for 24 h at 4 °C with a primary mouse anti-human TNF monoclonal antibody (Genzyme) diluted 1:100 in PBS containing 0.3% Triton X-100, 0.1% BSA, and 3% normal goat serum. Washed sections were incubated for 2 h with secondary biotinilated anti-mouse IgG (1:200, Vector Laboratories). The reaction product was visualized with 0.003% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine tetrahydrochloride as a chromogen. Negative controls were incubated in the absence of primary antibodies (not shown). Slides were analysed on a light microscope (Olympus BX60) using a MetaMorth Imaging System (Universal Imaging).

Animal model of endotoxic shock

Adult male Lewis rats (280–300 g, Charles River) were housed at 22 °C on a 12 h light/dark cycle. All animal experiments were performed in accordance with the National Institute of Health Guidelines under the protocols approved by the Institutional Animal Care and Use Committee of North Shore University Hospital/ New York University School of Medicine. Rats were anaesthetized with urethane (1 g kg^{-1}, intraperitoneally), and the trachea, the common carotid artery, and the jugular vein were cannulated with polyethylene tubing (Clay Adams). The catheter implanted into the right common carotid artery was connected to a blood pressure transducer and an Acquisition System (MP100, BIOPAC Systems) for continuous registration of mean arterial blood pressure (MABP). Animals were subjected to bilateral cervical vagotomy (VGX, $n = 7$) alone or with electrical stimulation (VGX+STIM, $n = 7$) or sham surgery (SHAM, $n = 7$). In vagotomized animals, following a ventral cervical midline incision, both vagus trunks were exposed, ligated with a 4-0 silk suture, and divided. In sham-operated animals both vagal trunks were exposed and isolated from the surrounding tissue but not transected. Electrical stimulation of the vagus nerve was performed in vagotomized animals. In these groups, the distal end of a vagus nerve trunk was placed across bipolar platinum electrodes (Plastics One) connected to a stimulation module (STM100A, Harvard Apparatus) and controlled by an Acquisition System. Constant voltage stimuli (5 V, 2 ms, 1 Hz) were applied to the nerve for 20 min (10 min before LPS administration and 10 min after). LPS (*Escherichia coli* 0111:B4; Sigma; 10 mg ml^{-1} in saline) was sonicated for 30 min, and administered at a lethal dose (15 mg kg^{-1}, intravenously). Blood was collected from the right carotid artery 1 h after LPS administration. Serum TNF amounts were quantified by the L929 bioactivity assay. To determine liver TNF amounts, animals were killed and livers rapidly excised, rinsed of blood, homogenized by polytron (Brinkman) in homogenization buffer (PBS, containing 0.05 % sodium azide, 0.5% Triton X-100 and a protease inhibitor cocktail; pH 7.2; 4 °C), and then sonicated for 10 min. Homogenates were centrifuged at 12,000*g* for 10 min, and TNF amounts in supernatants determined by ELISA (Biosource International). Liver TNF content was normalized to the amount of protein in the sample measured by the Bio-Rad protein assay.

Statistical analysis

Data shown are mean ± s.e.m. Statistical analyses between groups were performed using the two-tailed Student's *t*-test; P values < 0.05 were considered significant.

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Targeted destabilization of HY5 during light-regulated development of *Arabidopsis*

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Arabidopsis **seedlings display contrasting developmental patterns depending on the ambient light. Seedlings grown in the light develop photomorphogenically, characterized by short hypocotyls and expanded green cotyledons. In contrast, seedlings grown in darkness become etiolated, with elongated hypocotyls and closed cotyledons on an apical hook. Light signals, perceived by multiple photoreceptors and transduced to downstream regulators, dictate the extent of photomorphogenic development in a quantitative manner. Two key downstream components, COP1 and HY5, act antagonistically in regulating seedling development[1]. HY5 is a bZIP transcription factor that binds directly to the promoters of light-inducible genes, promoting their expression and photomorphogenic development[2,3]. COP1 is a RING-finger protein with WD-40 repeats whose nuclear abundance is negatively regulated by light[4,5]. COP1 interacts directly with HY5 in the nucleus to regulate its activity negatively[1]. Here we show that the abundance of HY5 is directly correlated with the extent of photomorphogenic development, and that the COP1–HY5 interaction may specifically target HY5 for proteasome-mediated degradation in the nucleus.**

To characterize the nuclear HY5 protein[2,3] biochemically, we produced rabbit polyclonal antibodies (anti-HY5) against recombinant HY5 protein. Western blot analyses of total protein extracts from light-grown wild-type seedlings revealed that anti-HY5 recognizes a protein band at an apparent relative molecular mass of 30,000 (M_r 30K) (Fig. 1a). The apparently large size of HY5 relative to its predicted size of 18K (ref. 2) is largely due to abnormal migration of the HY5 protein in our polyacrylamide gel system (data not shown). The presence of a 30K band in wild-type seedlings but not in the three *hy5* mutant alleles examined indicates that it is the endogenous *Arabidopsis* HY5.

Quantitative western blot analyses indicated that HY5 is 15–20 times more abundant in seedlings grown in white light than in those grown in the dark (Fig. 1b). This difference is observed in green tissues (cotyledon and hypocotyl) as well as in the non-photosynthetic roots (data not shown). We examined HY5 levels during light–dark transitions. Wild-type seedlings were grown in continuous light or darkness for four days and then transferred to the opposite light condition for 5, 10, 15 or 20 h. A western blot of the protein extracts from these seedlings revealed that although changes in the abundance of HY5 occur within 5 h, a full day of light or darkness is required for HY5 to reach its maximum or minimum, respectively (Fig. 1c).

Consistent with a previous report[2], HY5 messenger RNA levels

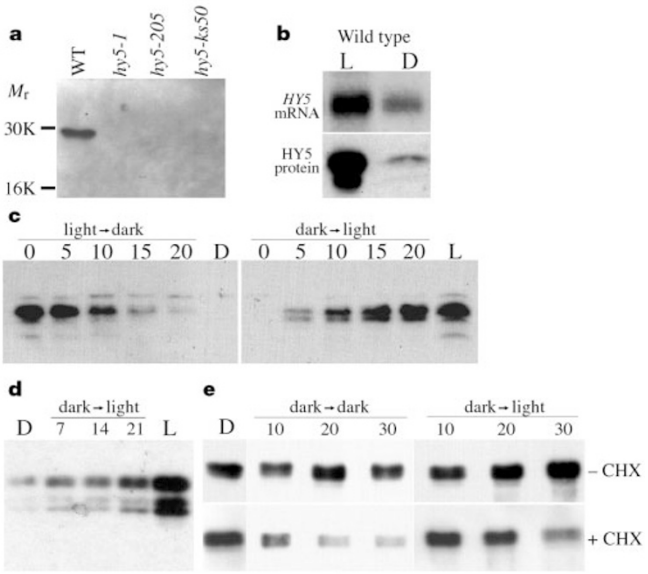

Figure 1 HY5 expression is regulated by light at both the mRNA and protein levels. **a**, A western blot of wild-type (WT), *hy5-1*, *hy5-205* and *hy5-ks50* seedling extracts using anti-HY5. **b**, A *HY5* northern blot and an anti-HY5 western blot of light- and dark-grown seedlings (L, light; D, dark). **c**, Anti-HY5 western blots from seedlings grown in continuous light (L) or darkness (D) for 4 d and then transferred to the opposite light condition for 5, 10, 15 or 20 h. **d**, An anti-HY5 western blot of 35SHY5 seedlings grown in continuous darkness for 4 d and then transferred to light for 0 (D), 7, 14 or 21 h. Seedlings grown in continuous light were included as a positive control (L). **e**, Anti-HY5 western blots of 35SHY5 seedlings treated with cycloheximide (+CHX) or buffer only (–CHX). Seedlings were grown in continuous darkness for four days before treatment and then transferred to light or darkness. Protein extracts were made 0, 10, 20 and 30 h after the addition of cycloheximide.