BBA - Bioenergetics 1860 (2019) 478–487

Contents lists available at ScienceDirect

BBA - Bioenergetics

journal homepage: www.elsevier.com/locate/bbabio

Mechanism and impact of catecholamine conversion by *Vibrio cholerae*

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ARTICLE INFO

Keywords:
Vibrio cholerae
Na^+ − NADH:quinone oxidoreductase
NQR
Superoxide
Adrenaline
Adrenochrome

ABSTRACT

Bacterial pathogens are influenced by signaling molecules including the catecholamines adrenaline and noradrenaline which are host-derived hormones and neurotransmitters. Adrenaline and noradrenaline modulate growth, motility and virulence of bacteria. We show that adrenaline is converted by the pathogen *Vibrio cholerae* to adrenochrome in the course of respiration, and demonstrate that superoxide produced by the respiratory, Na^+ − translocating NADH:quinone oxidoreductase (NQR) acts as electron acceptor in the oxidative conversion of adrenaline to adrenochrome. Adrenochrome stimulates growth of *V. cholerae*, and triggers specific responses in *V. cholerae* and in immune cells. We performed a quantitative proteome analysis of *V. cholerae* grown in minimal medium with glucose as carbon source without catecholamines, or with adrenaline, noradrenaline or adrenochrome. Significant regulation of proteins participating in iron transport and iron homeostasis, in energy metabolism, and in signaling was observed upon exposure to adrenaline, noradrenaline or adrenochrome. On the host side, adrenochrome inhibited lipopolysaccharide-triggered formation of TNF-α by THP-1 monocytes, though to a lesser extent than adrenaline. It is proposed that adrenochrome produced from adrenaline by respiring *V. cholerae* functions as effector molecule in pathogen-host interaction.

1. Introduction

Vibrio cholerae, the agent of cholera disease, harbours as primary electrogenic sodium pump, the Na^+ − translocating NADH:quinone oxidoreductase (NQR) [1]. This respiratory enzyme has no mammalian homologue and is broadly spread across pathogenic bacteria, including *Chlamydia trachomatis*, *Yersinia pestis*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa* [2,3]. *V. cholerae* does not harbor a mitochondrial complex I homologue like NDH-1, which is known from *E. coli*. The NQR, crucial for membrane energization and sodium transport, is considered to be a non-traditional drug target [4]. Deletion of the NQR leads to metabolic reprogramming and results in fermentation like metabolism, despite aerobic growth [5].

The NQR couples the exergonic oxidation of NADH with ubiquinone (Q_8) to the endergonic translocation of sodium ions from the cytoplasm to the periplasm [6,7] and thereby generates a sodium motive force [8]:

$$NADH + H^+ + Q_8 + 2Na^+_{cytoplasm} \rightarrow NAD^+ + Q_8H_2 + 2Na^+_{periplasm}$$

The three-dimensional structure of the NQR revealed the position of its redox cofactors (four flavins, two iron centers) in the multisubunit (NqrA-F) protein complex [1]. Electron transfer from NADH to Q_8 is described by the following scheme [8]:

$$FAD_{NqrF} \rightarrow 2Fe\text{–}2S_{NqrF} \rightarrow FeS_{NqrE/D} \rightarrow FMN_{NqrC} \rightarrow FMN_{NqrB} \rightarrow RBF_{NqrB}$$

During *in vivo* NADH oxidation, the reaction of O_2 with flavins exposed to the cytoplasmic (FAD_{NqrF}) and the periplasmic (FMN_{NqrB}) side of the complex, was accompanied with the release of superoxide ($O_2^{\bullet-}$) in both compartments [9,10].

The mammalian stress hormones adrenaline (A) and noradrenaline (NA) are important regulators of host physiology and behaviour, modulators of the outcome of a bacterial infection and actors in bidirectional communication between the host and its pathogen [11–13]. At the same time those catecholamines are prone to oxidation [14–16]. Adrenaline oxidation is a stepwise process: first, adrenaline-ortho-semiquinone is formed, followed by oxidation to adrenaline-ortho-quinone. After a cyclization to leukoadrenochrome, leukoadrenochrome-ortho-semiquinone is formed, which is then oxidized to adrenochrome (AC). Adrenochrome might rearrange to adrenolutin or

Abbreviations: A, adrenaline; NA, noradrenaline; AC, adrenochrome; NQR, Na^+- translocating NADH:quinone oxidoreductase

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https://doi.org/10.1016/j.bbabio.2019.04.003
Received 5 February 2019; Received in revised form 2 April 2019; Accepted 10 April 2019
Available online 12 April 2019

might be converted to non-soluble adrenomelanine [17]. In total, four electrons are abstracted from adrenaline to form adrenochrome. This oxidative conversion is accelerated in the presence of superoxide [18,19]. The arising oxidation products like adrenochrome were discussed to play a role in cardiotoxicity [20] or during septic shock [21]. Adrenochrome occurrence was reported after treating a patient suffering from septic shock with adrenaline, and was shown to contribute to myocardial necrosis [20]. To our knowledge adrenochrome has not yet been investigated concerning to its role in immune modulation or bacterial infection.

Adrenaline and noradrenaline modulate growth, motility and virulence of several Gram-negative bacteria including *Escherichia coli* and *Salmonella enterica* [11,22]. Despite growing interest in inter-bacterial [23] and pathogen-host interaction [24], information about the influence of human stress hormones on *V. cholerae* is limited [25]. We previously reported that adrenaline stimulates growth of *V. cholerae* in a serum-based medium. In supernatants from these *V. cholerae* cells, we observed partial, oxidative degradation of adrenaline to a compound which was proposed to represent adrenochrome [25]. The present study describes the mechanism of adrenochrome formation from adrenaline by *V. cholerae*. Superoxide produced by NQR during respiration acts as electron acceptor for the oxidation of adrenaline to adrenochrome. Once adrenochrome is formed, it modulates growth and proteome composition of *V. cholerae* and impacts TNF-α release of monocytic cells. We propose that besides adrenaline, adrenochrome acts as an effector molecule which is sensed by *V. cholerae*.

2. Material and methods

2.1. Production of V. cholerae membranes and purification of NQR

V. cholerae O395 N1 [26] and *V. cholerae* O395 N1Δ*nqr* [27] membranes were prepared [28] and NQR was purified as described [1,29,30]. Protein concentration was determined by the bicinchoninic acid method [31].

2.2. Enzymatic assays

Enzymatic assays were performed at 30 °C in 1 ml reaction buffer containing 20 mM TrisHCl pH 8, 100 mM NaCl, varying concentrations of adrenaline or adrenochrome and 5% (w/v) glycerol (for assays with membranes) or 0.05% DDM (for assays with purified NQR). Silver inhibition experiments were performed in 20 mM $Tris_2SO_4$ pH 8, 100 mM Na_2SO_4, 5% (w/v) glycerol and 5–10 μM $AgNO_3$. If indicated, the pH of the assay buffer was adjusted to 6 or 7 with NaOH in 20 mM BisTris, or to pH 9 with HCl in 20 mM CHES. The reaction was started with either 0.1 mM NADH, 0.5 μg purified NQR or 10–16 μg membranes. If indicated, 75 μM ubiquinone-1 was added to the reaction mixture. For anoxic experiments cuvettes sealed with a septum were flushed with N_2 and filled with reaction buffer, which also had been flushed with N_2. Changes in absorbance were monitored at 340 nm (NADH $\varepsilon_{\lambda 340} = 6.22\,mM^{-1}\,cm^{-1}$) and 480 nm (adrenochrome $\varepsilon_{\lambda 480} = 4.02\,mM^{-1}\,cm^{-1}$) [32] using a diode array spectrophotometer (Hewlett Packard) mounted with a magnetic stirrer.

2.3. Cultivation of V. cholerae

V. cholerae cells were grown on LB agar over night at 37 °C. A single colony was used to inoculate 5 ml LB medium ($10\,g\,l^{-1}$ tryptone, $5\,g\,l^{-1}$ yeast extract, $1\,g\,l^{-1}$ NaCl) supplemented with $50\,\mu g\,ml^{-1}$ streptomycin. Cells were grown overnight at 30 °C or 37 °C under shaking (180 rpm, Infors HT Ecotron). The cell pellet, obtained by centrifugation (3 min, 10,000 g), was washed and resuspended in glucose minimal medium [25] ($7\,g\,l^{-1}$ Na_2HPO_4, $3\,g\,l^{-1}$ KH_2PO_4, $0.5\,g\,l^{-1}$ NaCl, 18.7 mM NH_4Cl, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$, $40\,mg\,l^{-1}$ each of methionine, threonine, histidine, leucine and $2\,mg\,l^{-1}$ thiamine with 0.2% (w/v) glucose as a carbon source at pH 7.2) to obtain a cell suspension with a normalized OD_{600} of 2. This cell suspension was used as inoculum for the growth experiments described below. Adrenaline, noradrenaline and adrenochrome (Sigma-Aldrich) contained trace amounts of Fe as determined by ICP-MS in duplicates, which led to an increase in the iron concentration of the minimal medium by 6.2 ± 0.52 nM (adrenaline), 1.02 ± 0.04 nM (noradrenaline) or 0.49 ± 0.09 nM (adrenochrome) (n = 3) when compared to medium without added catecholamines.

The effect of adrenaline (100 μM) or adrenochrome (20–100 μM) on growth was followed in microtiter plates (1 ml wells) at 37 °C under shaking in a Tecan F200 pro plate reader at 595 nm. Glucose minimal medium was inoculated with a starting OD_{600} of 0.05.

To follow the fate of adrenaline and adrenochrome during growth of *V. cholerae*, 40 ml glucose minimal medium with 0.1 mM adrenaline was inoculated (OD_{600} = 0.02 at t = 0) and cells were grown at 30 °C under shaking in Erlenmeyer flasks. After 24 h cell-free supernatants were subjected to adrenaline and adrenochrome analysis by LC-MS.

To study the influence of adrenaline (0.1 mM), noradrenaline (0.1 mM) or adrenochrome (20 μM) on the proteome of the *V. cholerae* reference strain (termed WT), 15 ml glucose minimal medium was inoculated (OD_{600} = 0.05 at t = 0), and cells were grown at 37 °C under shaking in Erlenmeyer flasks. To obtain a reference proteome *V. cholerae* was grown without added catecholamines or adrenochrome. After 5–6 h (OD_{600} = 0.2–0.3), aliquots were taken and subjected to proteome analysis [5]. For each strain and each condition, three biological replicates were analyzed.

2.4. Confirmation of adrenaline and adrenochrome by LC-MS

Supernatants from growing cultures or from enzymatic assays were filtered (0.2 μm) and subjected to high performance liquid chromatography (HPLC) and ultra-high resolution tandem mass spectrometry. HPLC analysis was performed on an Agilent Technologies 1200 HPLC system with CatA eluent [25] at $0.6\,ml\,min^{-1}$ using isocratic flow. UV-VIS detection at fixed wavelengths (279 nm, 305 nm and 480 nm) and characteristic retention times allowed identification of fractions containing adrenaline and adrenochrome. HPLC electrospray ionization (ESI) mass spectrometry (MS) was used for further analysis of adrenaline and its derivate adrenochrome. The HPLC ESI-MS system consisted of an Agilent 1260 HPLC including a multiple wave length detector (Agilent Technologies) coupled to a maXis HD™ - UHR-TOF Mass Spectrometer (Bruker Daltonics). Please note that the extracted ion chromatogram is associated with a delay of approx. 30 s with respect to the UV-trace. Samples (20 μl) were injected onto a reversed-phase ProntoSIL analysis column (Bischoff, 120–3-C18 AQ, 250 mm × 4 mm (ID), 3 μm particle size) equipped with a ProntoSIL: 120–3-C18 AQ, 10 mm × 4 mm (ID) precolumn. Catecholamine phase A eluent at pH 4.3 was used as mobile phase [25]. Isocratic elution was performed at 30 °C as follows: flow rate at $300\,\mu l\,min^{-1}$ for 1 min, flow rate at $500\,\mu l\,min^{-1}$ for 5.8 min, and a total running time of 8.5 min.

For ESI-Q-TOF-MS analysis the electrospray ion source was operated in positive ionization mode. The capillary voltage was set to 4 kV. The desolvation gas (nitrogen) was heated to 180 °C at a flow rate of $8\,l\,min^{-1}$. The APCI (Agilent) tuning mix was used for referencing and the mass resolution was 40,000. Elemental compositions were determined by accurate mass measurements with standard deviations < 7.4 ppm. The HPLC-ESI-MS system was controlled by the "Hystar" software and UV-VIS and MS data were analyzed using the software "Data Analysis" (Bruker Daltonics).

2.5. Comparative proteome analysis

The proteome analysis was performed as described [5] and modified as follows: Only proteins identified with at least two peptides and LFQ (label-free quantification) values in all 3 replicates within one

condition, were considered for LFQ. Missing LFQ values were replaced by 22.5, the lowest detected LFQ intensity in the data set. Proteins with a *P*-value < 0.5 calculated by analysis of variance (ANOVA) and a ratio (RF, regulation factor) > ± 1.5 between treated samples and control samples were defined as differentially abundant. For those proteins with missing values in one condition no regulation factor was calculated, but changes in protein abundances were indicated as "up" and "down". Function assignment of proteins was performed by UniProt [33], KEGG [34–36] and NCBI databases [37,38]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [39] partner repository with the dataset identifier PXD009379.

2.6. Manipulation of THP-1 cells and TNF-α detection

Human monocytic THP-1 cells (DSMZ no.: ACC 16) were maintained at a cell density of 0.1–1 × 10^6 cells ml^{-1} in T25/T75 flasks in 90% RPMI supplemented by 10% heat-inactivated fetal calf serum (FCS) at 37 °C and 5% CO_2. To assess the effect of adrenaline and adrenochrome on cytokine production, 5 × 10^5 THP-1 cells were seeded per well of a 24-well plate and stimulated with 100 ng ml^{-1} lipopolysaccharide (*E. coli* O11:B4) without addition of any agent or in the presence of either 1, 10, 100, 10^3, 10^4 and 10^5 nM adrenochrome or 1, 10 and 100 nM adrenaline for 4 h at 37 °C and 5% CO_2. Negative controls comprised unstimulated THP-1 cells with or without addition of either 100 nM adrenochrome or 100 nM adrenaline. Subsequently, the supernatant was collected after centrifugation of the plates at 300 *g*, 4 °C and stored at −20 °C until further processing. Subsequently, all cells were resuspended in 100 µl 1xPBS. The total numbers of viable and dead cells were evaluated by staining with Gibco™ Trypan Blue Solution (Fisher Scientific GmbH) and by cell counting using a Neubauer-improved counting chamber. Concentration of TNF-α in supernatants was analyzed in triplicates according to manufacturer's recommendations by use of the Human TNF-alpha ELISA MAX™ Deluxe (BioLegend). Statistical analysis were performed with either IBM SPSS Statistics 24 or GraphPad Prism® 7 (GraphPad Software). For comparison of TNF-α concentration in the supernatant of LPS-stimulated THP-1 cells treated without agent or with different amounts of adrenaline and adrenochrome a Friedman-test was performed, taking into account small sample sizes and replicate-related effects on the maximal magnitude of TNF-α secretion by THP-1 cells. For subsequent post-hoc testing of stimulated THP-1 cells supplemented with the various amounts of adrenaline or adrenochrome against the control without addition of any agent (positive control) Dunn's multiple comparison test was performed. The half maximal inhibitory concentration (IC_{50}) of either adrenaline or adrenochrome on the TNF-α secretion of THP-1 cells was assessed separately for every replicate by nonlinear regression analysis using GraphPad Prism 7. In detail, a nonlinear curve with four-parameter variable slope was fitted to the obtained TNF-α concentrations (pg/ml, y-coordinate) at particular concentrations of adrenaline or adrenochrome (nM, x-coordinate) by use of the least squares fit method and the constraints a) top of curve set to pg/ml TNF-α in positive control and b) bottom of curve set to 0 pg/ml TNF-α. Gaussian distribution was confirmed by Shapiro-Wilk normality test of residuals and homoscedasticity by plotting residuals versus predicted values. Only IC_{50} obtained from fitted curves resulting in $R^2 > 0.84$ (n = 7 with $R^2 > 0.92$, n = 3 with $R^2 = 0.84–0.9$) were used for further statistical comparison of IC_{50}-values between adrenaline and adrenochrome by a two-tailed Wilcoxon signed-rank test. To assess a potential association of the various culture conditions with the number of dead THP-1 cells in culture, probably displaying an underlying mechanism for altered TNF-α secretion of THP-1 cells, the data were analyzed with linear mixed models (LMM, MIXED command, Type III sums of squares). The restricted maximum likelihood method (REML) was used for parameter estimation. The intercept, the factors LPS (yes/no) and type of agent (none/ adrenaline/ adrenochrome), as well as the covariate amount of agent (nM) were set as fixed effects. Replicate (1–5) was set as random effect with a scaled identity (ID) covariance structure. A natural logarithmic transformation of the number of dead cells was necessary to meet homoscedasticity and normality, which were confirmed by plotting residuals versus predicted values and by quantile-quantile plots of residuals, respectively. The LMM was followed by post-hoc multiple testing with Bonferroni correction to compare the number of dead THP-1 cells in culture by addition of no agent, adrenaline or adrenochrome.

3. Results

3.1. The NQR provides superoxide as electron acceptor for the oxidation of adrenaline to adrenochrome

We hypothesized that respiratory O_2 reduction might affect adrenochrome formation, and therefore studied the conversion of adrenaline by membranes from *V. cholerae*. The respiratory chain of the inner membrane of *V. cholerae* starts with a the NADH:quinone oxidoreductase (NQR) which transports Na^+ but not H^+ [1]. This redox pump represents the first, energy generating segment of the respiratory chain of *V. cholerae*. Besides NQR, *V. cholerae* possesses a non-electrogenic NADH dehydrogenase (NDH-2), which permits survival of *V. cholerae* mutant devoid of NQR [9]. Quinol, formed by either NQR or NDH-2, is oxidized by downstream respiratory enzymes under ultimate reduction of O_2 to H_2O by the terminal oxidase of *V. cholerae*. We followed adrenochrome formation from adrenaline using membranes from the *V. cholerae* reference strain (WT) exposed to NADH as electron donor. In the presence of O_2 (Fig. 1A), increase in absorbance at 480 nm indicated formation of adrenochrome, as confirmed by UV-VIS spectroscopy (Fig. 1A, inset) and mass spectrometric analysis of the assay mix at the end of the reaction (Fig. S1). No adrenochrome formation was observed without membranes in the presence of NADH, or with membranes from the *V. cholerae* mutant devoid of NQR (Fig. 1A). Next, we repeated the experiments under exclusion of O_2. No adrenochrome was formed by membranes from the reference strain when NADH was added (Fig. 1B). Taken together, we conclude that adrenochrome formation by *V. cholerae* membranes requires the NQR, NADH and O_2. Further proof that the NQR is responsible for adrenochrome formation was obtained by decreasing NQR activity with Ag^+ (Fig. 2A). Ag^+ irreversibly inhibits initial NADH oxidation catalyzed by subunit NqrF [40], and at the same time, adrenochrome formation was clearly diminished. With NADH as electron donor, mitochondrial complex I is capable of reducing adrenochrome instead of ubiquinone, using adrenochrome as an alternative electron acceptor [41,42]. We tested if membranes from *V. cholerae* WT reduce adrenochrome with NADH as electron donor, but found no evidence for the reduction of adrenochrome by the NQR from *V. cholerae* (Fig. 1C).

We also asked if noradrenaline might be converted to noradrenochrome by *V. cholerae* membranes oxidizing NADH in the presence of O_2. An increase in absorbance at 480 nm indicated formation of noradrenochrome, but with a decreased rate when compared to adrenochrome formation (Fig. 2B).

Fridovich and coworkers [18] reported that superoxide serves as electron acceptor for initial oxidation of adrenaline to its semiquinone radical. The semiquinone is likely to undergo disproportionation. The disproportionation rate is low at alkaline pH and higher at acidic pH [19]. Thus, alkaline pH enhances the net formation of ortho-semiquinone, as confirmed under our experimental assay conditions (Fig. S2). To estimate the impact of pH on adrenochrome formation, one must also consider the pH dependency of electron transfer activity of NQR, which is highest at pH 7.5 to 8 and decreases by around 40% at pH 6.5 [43]. This will contribute to increased rates of adrenochrome formation at pH 8 compared to pH 6–7 (Fig. S2A–B). At pH 9 adrenolutin rather than adrenochrome is formed (Fig. S2C). However, this conversion at very alkaline conditions is not a relevant process within

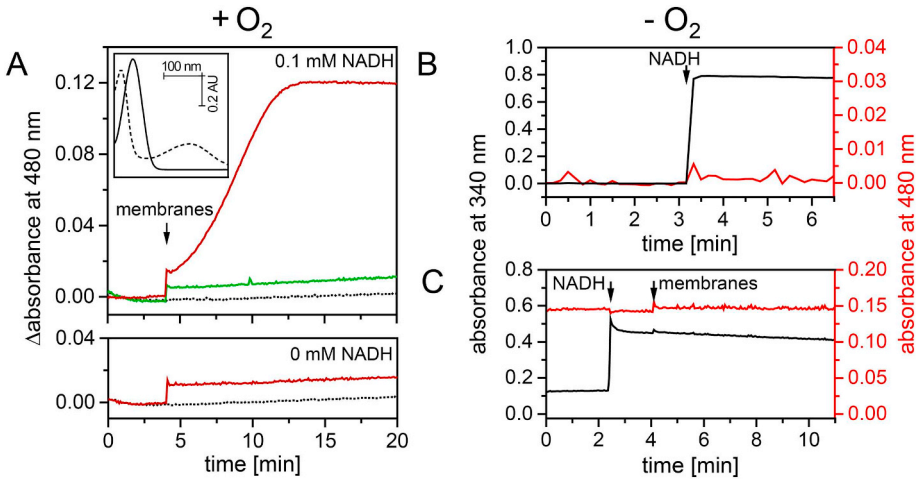

Fig. 1. Conversion of adrenaline to adrenochrome by *V. cholerae* membranes requires NADH, O_2 and the respiratory NADH:quinone oxidoreductase (NQR). (A) Air saturated assay buffer containing 0.1 mM adrenaline with (upper panel) or without (lower panel) 0.1 mM NADH. Assays were performed with membranes (10 µg) from *V. cholerae* WT (red trace) or the *nqr* deletion strain (green trace). In control reactions (dotted traces), membranes were omitted. The formation of adrenochrome was monitored by the increase of absorbance at 480 nm. Inset shows spectra (290–590 nm) recorded before (solid line) and after 20 min (dashed line) of adrenochrome formation. (B and C) assays with anoxic buffers. In panel B, membranes from *V. cholerae* WT were mixed with 0.1 mM adrenaline. 0.1 mM NADH was added and the absorbance at 340 nm (NADH, black) and 480 nm (adrenochrome, red) was recorded. In panel C, assay buffer contained 25 µM adrenochrome. Upon addition of 0.1 mM NADH and further addition of 10 µg membranes from *V. cholerae* WT, absorbances at 340 nm (black) and 480 nm (red) were recorded.

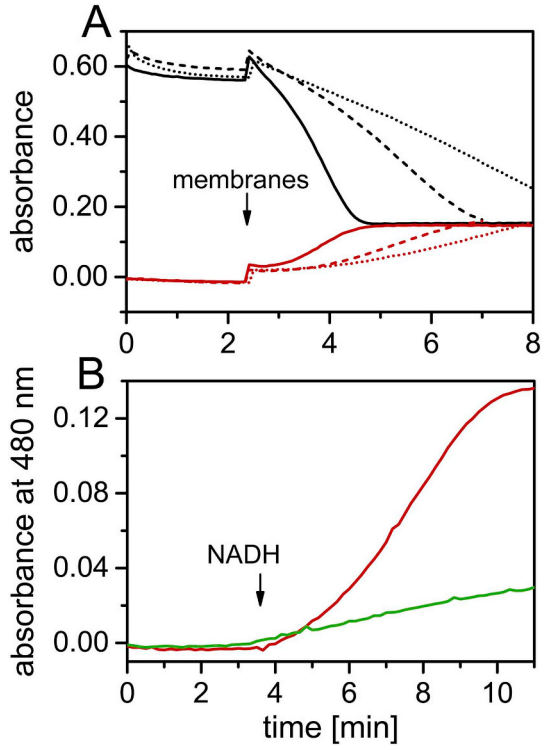

Fig. 2. Inhibition and specificity of catecholamine conversion by *V. cholerae* membranes. (A) Membranes (16 µg) with 0.1 mM adrenaline, 0.1 mM NADH and increasing concentrations of $AgNO_3$ (5 µM, dashed line; 10 µM, dotted line). Solid line, no $AgNO_3$ added. Reaction was started with membranes, oxidation of NADH (black traces) and formation of adrenochrome (red traces) were followed. (B) Conversion of 0.1 mM adrenaline (red trace) or 0.1 mM noradrenaline (green trace) by membranes (10 µg) upon addition of 0.1 mM NADH.

the gastro intestinal tract of the human host. We hypothesized that superoxide formed by NQR as a side-product during oxidation of NADH [9,10] represents the catalytic intermediate for the observed NADH-dependent conversion of adrenaline to adrenochrome by the NQR. Using purified NQR solubilized in detergent in absence of its terminal electron acceptor ubiquinone, we confirmed adrenochrome formation from adrenaline upon addition of NADH in an air-saturated assay (Fig. 3A). If superoxide dismutase was added after a first pulse of NADH, a second pulse of NADH did not result in adrenochrome formation (Fig. 3B). In the presence of ubiquinone-1, the rate of NADH oxidation increased, and adrenochrome formation was accelerated (Fig. 3D). This was caused by increased superoxide formation caused by the reaction of ubisemiquinone with O_2 [44]. Without NADH, NQR did not catalyze adrenochrome production from adrenaline (Fig. 3C). This demonstrates that $O_2^{\bullet-}$ formed by NQR acts as an electron acceptor for oxidation of adrenaline to adrenochrome. At high (0.5 mM) concentrations of adrenaline, the ratio of NADH oxidized/adrenochrome formed reaches 2 (Fig. S3). Under these conditions, in the overall reaction, two molecules of NADH were oxidized by NQR to yield four molecules of $O_2^{\bullet-}$ from O_2, which acted as single electron acceptors for the four-electron oxidation of adrenaline to adrenochrome (Fig. 4C). The ratio of the specific NADH oxidation and adrenochrome formation activities increased from 2.1 at 500 µM adrenaline to 73.1 at 1 µM adrenaline (Fig. S3). This was largely caused by the dramatic increase in adrenochrome formation rates from 0.01 µmol min^{-1} mg^{-1} (at 1 µM adrenaline) to 0.66 µmol min^{-1} mg^{-1} (at 500 µM adrenaline). At the same time rates of NADH oxidation increased only 2.2-fold, from 0.61 µmol min^{-1} mg^{-1} (at 1 µM) to 1.37 µmol min^{-1} mg^{-1} (at 500 µM adrenaline). We conclude that under the specified conditions the rate of adrenochrome formation is not limited by $O_2^{\bullet-}$ production rates of NQR, but by the concentration of the reactant adrenaline in the non-enzymatic oxidation reaction of adrenaline with $O_2^{\bullet-}$.

3.2. Confirmation of adrenochrome formation by mass spectrometry

The characterization of adrenochrome in the supernatant of *V. cholerae* cells grown in presence of adrenaline, and in the reaction mix containing *V. cholerae* membranes, was achieved by HPLC-Electrospray Ionization (ESI) -mass spectrometry (Fig. 4A, B and Fig. S1). The identities of adrenaline and adrenochrome were confirmed by comparing retention times and mass spectra of samples to the reference compounds adrenaline and adrenochrome (Fig. S5). The derivative adrenochrome eluted as symmetrical peaks, yet earlier than its precursor adrenaline. Identification of adrenochrome in the samples was accomplished by high resolution MS and visualized by an extracted ion chromatogram (EIC) of the corresponding ions (e.g. $[M + Na]^+$ at *m/z* 202 for adrenochrome). The precise m/z values of the ions were used to calculate the corresponding elemental composition CxHyNOz for each metabolite (Fig. S1, Table S1). Protonated adrenaline and adrenochrome are partially dissociating in the ion source during electrospray ionization and easily undergo water loss to form $[M + H\text{-}H_2O]^+$ ions

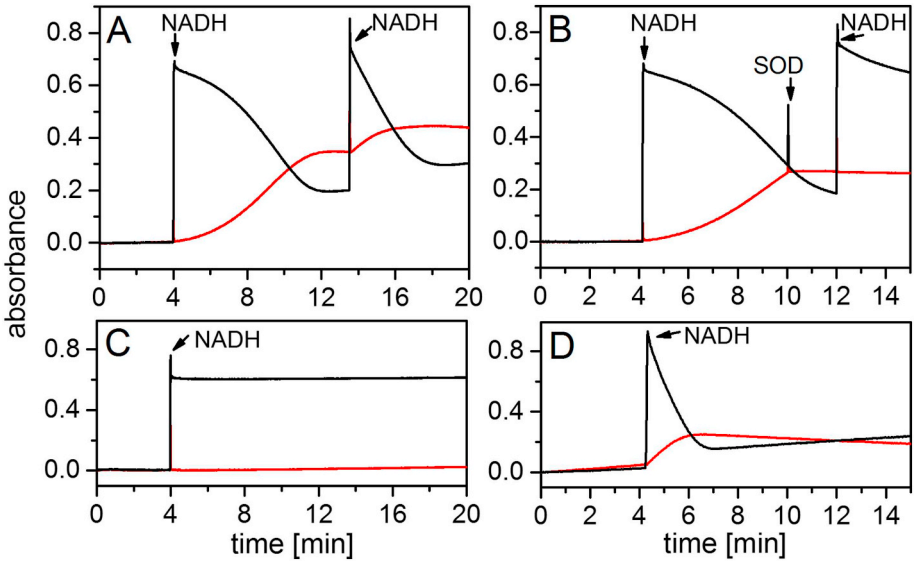

Fig. 3. Superoxide-dependent formation of adrenochrome by NQR. Purified NQR (0.5 μg) in detergent micelles was allowed to react with NADH in air saturated buffer with 1 mM adrenaline. Absorbance was detected at 340 nm (black) and 480 nm (red). (A) Two subsequent additions of NADH (each 0.1 mM). (B) Addition of superoxide dismutase (SOD, 100 U) inhibits adrenochrome formation. (C) Control without NQR. In (D), the assay mixture contained NQR, adrenaline and 75 μM ubiquinone-1. The reaction was started with NADH.

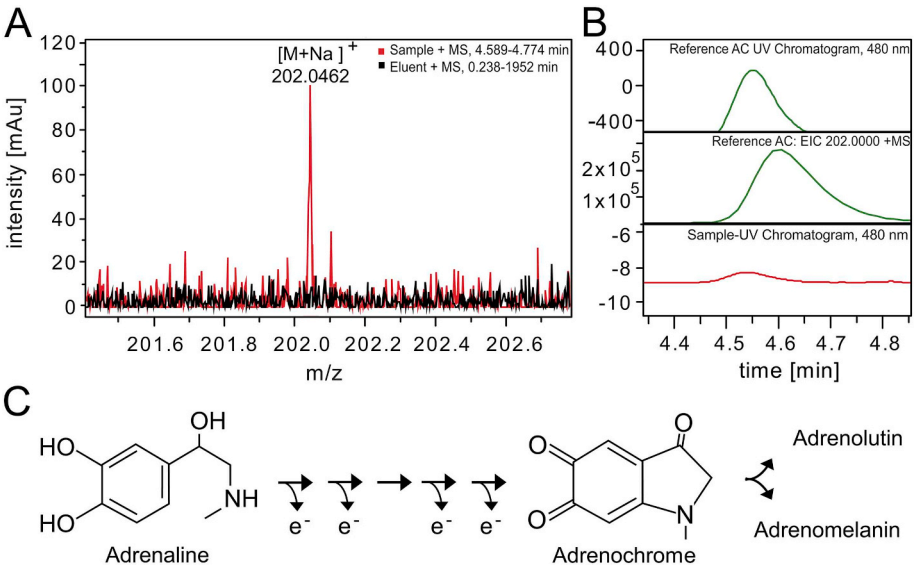

Fig. 4. Conversion of adrenaline to adrenochrome. (A) *V. cholerae* cells were exposed to 0.1 mM adrenaline in glucose M9 medium. Detection of adrenochrome at *m/z* 202 $[M + Na]^+$ (red) and a retention time of 4.59–4.78 min in supernatants of growing cells after 24 h. The intensity of the eluent is shown in black. (B) UV chromatogram at 480 nm (top) and LC-MS extracted ion chromatogram of m/z 202 $[M + Na]^+$ of the reference compound adrenochrome (AC, middle) together with the UV chromatogram at 480 nm (bottom) of the sample shown in A. (C) Scheme for oxidation of adrenaline to adrenochrome and further conversion to adrenolutin or adrenomelanin.

(m/z 166) having ca. 5-fold higher intensity than the $[M + H]^+$ ions [45]. Only adrenaline forms $[M + H]^+$ ions (m/z 189.09) at appreciable abundance. In case of adrenochrome, the Na^+-adduct is the dominating ion [46]. Given that *V. cholerae* is a strong producer of $O_2^{\bullet-}$ [9,10], and that activated macrophages produce superoxide, we can assume that NQR containing bacteria such as *V. cholerae* exposed to adrenaline or noradrenaline, will also be confronted with (nor)adrenochrome. This raised the question whether there would be specific responses of the pathogen and the host towards adrenochrome, addressed in the following sections.

3.3. Impact of adrenochrome on immune cells

We next compared the effect of adrenaline and adrenochrome on LPS-induced TNF-α production of the monocytic human cell line THP-1. Adrenaline is known to modulate the LPS-induced cytokine production of THP-1 cells via ß-adrenergic receptors [47,48]. When added simultaneously with LPS, we found inhibitory effects for both agents; although to a much lesser extent for adrenochrome compared to adrenaline (Fig. 5; $X^2(9) = 31.09$, $P = 0.0003$). Whereas 100 nM adrenaline already led to a significant reduction of TNF-α production within THP-1 cells compared to the positive control without addition of any agent ($P < 0.01$), at least 10 μM of adrenochrome was needed to obtain the same effect (Fig. 5A; $P = 0.0064$ for 100 nM adrenaline, $P = 0.0456$ for 10 μM adrenochrome, $P = 0.0216$ for 100 μM adrenochrome, respectively). Accordingly, the half maximal inhibitory concentration (IC_{50}) for adrenochrome (mean = 15,706 ± 6836 nM) was found to be two to three decades higher compared to the IC_{50} of adrenaline (mean = 21.23 ± 3.09 nM) (Fig. 5B; $Z = -2.023$, * $P = 0.04$). The differences in inhibition of TNF-α production of THP-1 cells of adrenaline and adrenochrome thereby did not rely on differences in the number of dead THP-1 cells in culture. Although linear mixed model analysis revealed an increasing effect on the number of dead cells within the culture by addition of adrenaline or adrenochrome (Table S2; $F(2,44) = 3.705$, $P = 0.033$), both agents did not differ in this effect (Fig. S4).

3.4. Impact of adrenochrome on Vibrio cholerae

As previously shown, *V. cholerae* also showed improved growth and motility when exposed to adrenaline in a medium containing native serum components [25]. In glucose minimal medium, adrenochrome as well as adrenaline stimulated growth of *V. cholerae*. The addition of 50 μM adrenochrome, or 100 μM adrenaline, enhanced the final optical

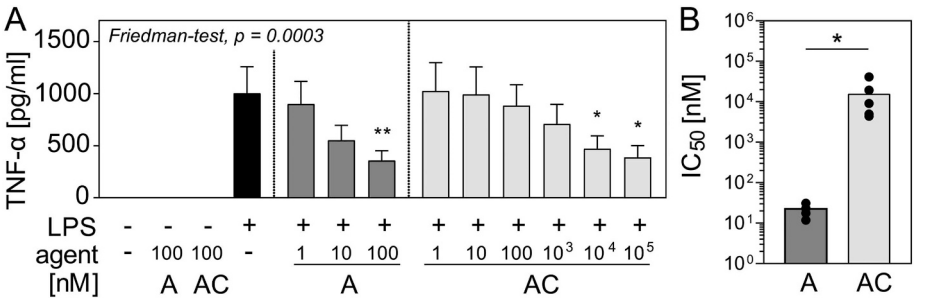

Fig. 5. Adrenochrome inhibits TNF-α production of THP-1 cells to a lesser extent than adrenaline. THP-1 cells were cultured in the presence of 100 ng/mL LPS and increasing concentrations of A (adrenaline) or AC (adrenochrome). (A) TNF-α concentration in supernatant of LPS-stimulated THP-1 cells with or without different amounts of adrenaline or adrenochrome (n = 4, mean +/− SEM); Friedman-test without unstimulated controls ($X^2(9) = 31.09$, $P = 0.0003$) followed by post-hoc testing against control without addition of any agent (positive control) with Dunn's multiple comparison test; asterisks indicate significant difference from positive control with * $P < 0.05$, ** $P < 0.01$. (B) Half maximal inhibitory concentration (IC_{50} in nM) of A (adrenaline) or AC (adrenochrome) on TNF-α secretion of LPS-stimulated THP-1 cells (n = 5, mean + single values), Wilcoxon-test, two-tailed ($Z = -2.023$, * $P = 0.04$).

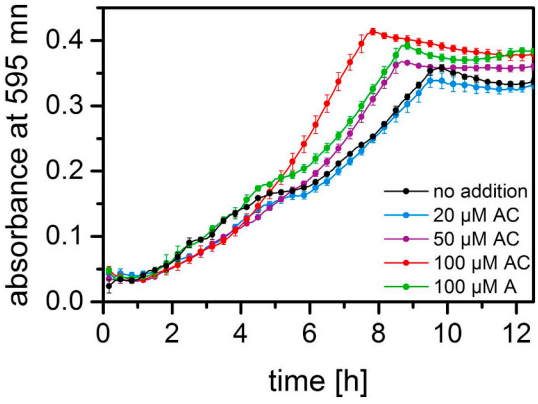

Fig. 6. Adrenochrome stimulates growth of *V. cholerae*. *V. cholerae* was grown aerobically in glucose-containing minimal medium in replicates (n = 3) of 1 ml. 20–100 µM AC (adrenochrome) or 100 µM A (adrenaline) were added. Mean values and standard deviations are shown.

density and the growth rate of *V. cholerae* (Fig. 6) to a similar extent, whereas addition of 20 µM adrenochrome showed no effect. Highest growth rates were observed with 100 µM adrenochrome.

We also analyzed the change in proteome of *V. cholerae* upon addition of adrenaline (A) or adrenochrome (AC, Table 1). Proteins changed in abundances upon adrenaline or adrenochrome exposure participate in iron homeostasis, metabolism, signaling or translational and transcriptional control (Table 1). The complete data set, including also the proteins not changed in abundance, has been deposited in the PRIDE database [39]. Iron is known to form complexes with catecholamines and adrenochrome [49–51] and it was reported that catecholamines enhance iron accessibility for bacteria by eliciting iron from host-associated chelators found in serum-based media [49,50]. The glucose minimal medium used here was not supplemented with iron, and did not contain host-associated chelators. The ferrous iron transporter FeoA (A0A0H3AM36) and FeoB (A0A0H3AFT4), which are located in the inner membrane were increased in abundance in *V. cholerae* upon addition of noradrenaline or adrenochrome, respectively. In contrast, a ferric iron ABC-transporter (A0A0H3AKY1) showed decreased abundance upon exposure to noradrenaline and adrenochrome. The vibriobactin- and enterobactin- specific ABC transporter ViuA showed decreased abundance in *V. cholerae* cells treated with adrenaline compared to the untreated control. We identified IrgA (Table 1, A5F9G0), an outer membrane receptor for the bacterial siderophore enterobactin [52,53] to be increased in abundance during growth of *V. cholerae* in the presence of adrenaline, noradrenaline and adrenochrome. Also, VctA (A0A0H3ADQ9), a TonB-dependent iron-siderophore transporter was slightly increased in abundance when *V. cholerae* was treated with adrenaline or noradrenaline. The results demonstrate that adrenaline, noradrenaline as well as adrenochrome influence iron acquisition in *V. cholerae*. Furthermore, we found a set of proteins without annotated function, which were changed in abundance upon catecholamine exposure. One of these proteins (A5F0B6) was increased in abundance in every tested condition (RF +2.0 to +5.4) and contains a YecI domain. In *Helicobacter pylori* the periplasmic YecI protein interacts with isoprenoid quinone and is upregulated under acidic pH and contributes to survival of the acidic stomach passage [54]. Noteworthy, during oxidation of catecholamine quinone intermediates are formed, but why this protein showed increased abundance in presence of adrenaline, adrenochrome and noradrenaline is not yet understood and needs further investigations. A strong shift in abundance of an uncharacterized protein (A0A0H3AK32) was also detected in *V. cholerae* during exposure to adrenaline, noradrenaline and adrenochrome. It is assumed from sequence information that this protein is a transport protein and mediates the efflux of not further characterized substances. The protein with highest increase in abundance (A0A0H3AE12, RF +14.85) was identified during exposure to adrenaline. It was also increased in abundance during exposure to adrenochrome (RF +7.64) and noradrenaline (RF +4.43). This protein has no functional annotation and is member of the DUF3316 family. Certain members of this family are believed to be acyl-CoA synthetases. When challenged with externally added adrenaline, noradrenaline, or adrenochrome, there was a profound response in the proteome profile of *V. cholerae* which was in some parts quite similar to the proteome shift of *V. cholerae* challenged with 0.35 mM externally added indole [55]. Indole represents the core chemical moiety of adrenochrome. Like adrenochrome, indole caused downregulation of *cheY*, *metE* and *hutG* as well as upregulation of VC1722 as shown in a transcriptome study [55].

Diminished abundance of HutG (A5F1X7) and MetE (A0A0H3AM91) upon treatment with adrenaline and noradrenaline was also observed. MetE contributes to the synthesis of L-methionine out of L-homocysteine, whereas HutG contributes to the degradation of L-histidine to L-glutamate. In contrast *irgA* showed downregulation by indole [55], but an upshift in abundance by adrenochrome, adrenaline and noradrenaline. Our results indicate that adrenochrome and the signaling molecule indole are perceived by *V. cholerae* in a comparable manner, with unknown but obviously related underlying signaling events.

Adrenaline and noradrenaline act as signaling molecules via specific adrenergic receptors in eukaryotes or bacteria. In *Escherichia coli* or *Salmonella enterica*, QseC was identified as bacterial, adrenergic receptor and QseB as a response regulator, controlling transcription of associated genes like virulence and flagellar genes [56,57]. Halang et al. discussed the function of a putative histidine kinase in *V. cholerae* with 29% identity and 47% similarity to QseC from *E. coli* [25]. This putative QseC homologue of *V. cholerae* was not detected to be changed in abundance upon treatment compared to the non-treated control. Neither flagellar proteins, nor virulence proteins were detected to be changed in abundance during exposure of adrenaline, adrenochrome or noradrenaline. A LysR-type regulator was decreased in abundance during exposure to adrenaline and adrenochrome in the wild type. However, a not further characterized response regulator

Table 1
Proteins in- or decreased in abundance upon adrenaline (A), noradrenaline (NA) or adrenochrome (AC) treatment during growth of *V. cholerae*. A positive regulation factor (RF) indicates an increase in protein abundance. *V. cholerae* protein IDs refer to the UniProt database. VC numbers are derived from the *V. cholerae* El Tor biotype. Functional assignment is based on UniProt and KEGG database annotation, refined by NCBI-based BLAST results. *N.a.*, not annotated. "Down" or "up" indicates an increase or decrease in protein abundance, in case where RF for the corresponding protein could not be determined since missing values were replaced by a constant.

Protein ID	VC number	Gene name	Assigned function	A	NA	AC
Iron Homeostasis						
A5F9G0	VC0475	*irgA*	Iron-regulated outer membrane receptor for ferrienterochelin and colicins, enterobactin receptor protein	+3.1	+4	+1.5
A0A0H3ADQ9	n.a.	*n.a.*	Enterobactin receptor VctA, hemoglobin/transferrin/lactoferrin receptor protein	+1.8	+1.8	
A0A0H3AM36	VC2078	*feoA*	Ferrous iron transport protein A		+2.2	
A0A0H3AFT4	VC2077	*feoB*	Ferrous iron transport protein B			+1.9
A0A0H3AKY1	VC0610	*n.a.*	Ferric iron ABC transporter, ATP-binding protein		down	down
A0A0H3ANY0	VC0779	*viuC*	Vibriobactin and enterobactin ABC transporter, ATP-binding protein	down		
Metabolism/energy						
A0A0H3AM91	VC1704	*metE*	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	−5.1	−2.8	−4.2
A5F1X7	VC1204	*hutG*	Formimidoylglutamase	−2.6	−1.9	−1.9
A0A0H3AFI4	VCA0984	*lldD*	L-lactate dehydrogenase	−1.7		−3.7
A0A0H3AE36	VCA0615	*msrB*	Peptide methionine sulfoxide reductase		−2.5	
A5F3I4	VC0384	*cysJ*	Sulfite reductase [NADPH] flavoprotein alpha-component	+2.1		
A0A0H3AES2	VCA0099	*n.a.*	Oxidoreductase, Gfo/Idh/MocA family			+2.0
A0A0H3AEX9	VCA0584	*n.a.*	Putative glutathione S-transferase	up	up	up
A0A0H3AML3	VC2710	*spoT*	Guanosine-3′,5′-bis(Diphosphate) 3′-pyrophosphohydrolase		up	up
A0A0H3AMY6	VC2613	*prkB*	Phosphoribulokinase		up	up
A5F697	VC2180	*hemA*	Glutamyl-tRNA reductase		up	up
A0A0H3AJU8	VC0648	*n.a.*	Lipoprotein NlpI		up	up
Signaling						
A0A0H3AL68	VC1082	*n.a.*	Response regulator		+2.6	+3.9
A0A0H3ADB6	VCA0068	*n.a.*	Methyl-accepting chemotaxis protein	+2.7		+2.2
A0A0H3ADY5	VCA1034	*n.a.*	Methyl-accepting chemotaxis protein		up	up
A0A0H3AJM9	VC0076	*uspA*	Universal stress protein		up	up
A0A0H3AE44	VCA1096	*cheY-4*	Chemotaxis protein CheY			down
A0A0H3ADQ8	VCA1055	*n.a.*	Transcriptional regulator, LysR family	down		down
Regulation of translation and transcription						
Q09HM8	VCA0198	*vchM*	Cytosine-specific DNA methyltransferase	up	up	up
A5F5C5	VC2506	*rapA*	RNA polymerase-associated protein RapA/ HepA	up	up	
A0A0H3AGT5	VCA0969	*n.a.*	Pirin family protein	up	up	
A0A0H3AJV0	VC2691	*cpxP*	Putative periplasmic protein CpxP	up		
			Chaperones and folding catalysts			
A0A0H3AMZ5	VC1722	*n.a.*	Uncharacterized protein: DNA transformation protein and related proteins			+2.4
A0A0H3AKJ1	VC0039	*n.a.*	Sporulation control - related protein SpoOM	+2.2		
Uncharacterized proteins with weak functional assignments						
A5F0B6	VCA0539	*n.a.*	UPF0312 protein with YceI-like domain	+5.4	+2.4	+2.0
A0A0H3AE12	VCA0139	*n.a.*	DUF3316 domain-containing protein	+14.9	+4.3	+7.6
A0A0H3AK32	VC0913	*n.a.*	Putative efflux transporter	+6.6	+4.3	+4.3
A0A0H3ANY3	VC2111	*n.a.*	Putative NAD(P)H-dependent FMN reductase	+4.8	+1.8	
A0A0H3AHH1	n.a.	*n.a.*	Mu-like prophage major head subunit gpT			+2.2
A0A0H3AGG2	n.a.	*n.a.*	Uncharacterized protein	up		up
A0A0H3AKC4	VC0163	*n.a.*	Uncharacterized protein	up	up	
A0A0H3AHL3	VC2552	*n.a.*	Uncharacterized protein		−5.0	
A0A0H3AGA3	VCA0631	*n.a.*	Putative acetyltransferase		down	

(A0A0H3AL68) was increased in abundance. Also, two chemotaxis proteins (A0A0H3ADB6 and A0A0H3ADY5) were increased in abundance, whereas the chemotaxis protein CheY (A0A0H3AE44) showed decreased abundance, but only in presence of adrenochrome. To our knowledge these chemotaxis and signaling related proteins are working independently from each other and do not cluster. The comparative proteome study did not reveal an obvious candidate for specific receptors or signaling pathways for neither adrenaline, noradrenaline or adrenochrome in *V. cholerae*, but these three molecules obviously are sensed and have an effect on the physiology of the pathogen.

4. Discussion

In the field of microbial endocrinology the influence of host derived (nor-)adrenaline on gastrointestinal bacteria is intensively studied [22,49,57]. It was shown, that many Gram-negative bacteria sense those hormones and respond to them with enhanced growth, virulence, biofilm formation or attachment. During ingestion and colonization of *V. cholerae* it is likely that *V. cholerae* faces those catecholamine hormones. Beside the gastro intestinal (GI) tract, a first site of interaction between bacteria and catecholamines are dental pulps, where occurrence of adrenaline and noradrenaline was described [58]. Interestingly also phagocytic cells were reported to be a source of catecholamine release during infection [59]. In the lumen of the human GI tract catecholamine levels were not yet determined, since this is still methodically challenging. Catecholamine concentration in the GI lumen, will presumably fluctuate wildly, since they are dependent on the distance to release and the rate of release, which also adapts to stimuli like stress level. However, within the ileum-lumen of germ-free and specific pathogen-free mice average concentration of 7.5 ng/g and 9 ng/g noradrenaline and traces of adrenaline were reported, respectively [60]. In the colon lumen noradrenaline levels were described to be 3.2 ng/g (germ-free mice) and 60.5 ng/g (specific pathogen-free mice). Further NQR-harbouring bacteria may come in contact with catecholamines in the urinary tract or respiratory tract or during sepsis (e.g. *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*). In human urine up to 80 µg

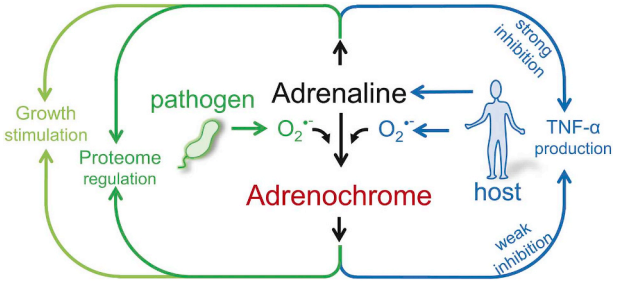

Fig. 7. Proposed scheme of adrenaline and adrenochrome interaction at the host pathogen interface. The endocrine hormone adrenaline is converted to adrenochrome (red) in presence of superoxide ($O_2^{\bullet-}$). $O_2^{\bullet-}$ is released either in respiratory processes of the pathogen (green) or by the immune system of the host (blue). Adrenaline as well as adrenochrome stimulate pathogen's growth in metal limited medium and affect the proteome. Adrenaline shows immune modulative capacity, by strongly inhibiting pro-inflammatory TNF-α production of THP-1 cells. Adrenochrome also inhibits monocytic TNF-α production but less pronounced than adrenaline.

noradrenaline and 20 µg adrenaline catecholamines were detected within 24 h [61]. In blood plasma catecholamine levels are in the lower nM range for both adrenaline (up to 0.7 nM) and noradrenaline (up to 10 nM) [61]. It is reasonable to assume that locally, at the site of release catecholamines may appear in µM concentrations. In contrast, catecholamine levels might decrease through oxidation.

In this study, we show that oxidation from adrenaline to adrenochrome is caused by superoxide derived from the respiratory chain of *V. cholerae*. We confirmed the occurrence of adrenochrome by LC-MS, and showed that the NADH:quinone oxidoreductase from *V. cholerae* is the source for superoxide dependent adrenaline oxidation. This observation raised the question, whether the oxidation product adrenochrome is sensed by bacterial and host cells and acts in a similar manner to adrenaline. The proteome of *V. cholerae* adjusts upon exposure to adrenaline, adrenochrome and noradrenaline. Adrenaline, adrenochrome and noradrenaline share the ability to alter iron homeostasis in *V. cholerae*. The catechol group of adrenaline and noradrenaline enables iron chelation [50] and adrenochrome was also discussed to have these properties [51]. When exposed to host iron chelating compounds like lactoferrin or transferrin, catecholamine-iron complexes release the host-bound iron and make it accessible for bacterial acquisition [49,50,62,63]. Here we show that *V. cholerae* enhances its catechol-siderophore receptor IrgA upon exposure to adrenaline, noradrenaline or adrenochrome. In *E. coli* the bacterial catechol-siderophore enterobactin is responsible for noradrenaline mediated iron uptake, acting as competitor for lactoferrin or transferrin and thus facilitating growth in an iron limited growth medium [62]. Although *V. cholerae* itself does not produce enterobactin but vibriobactin, *V. cholerae* possesses with IrgA and VctA two receptors for ferric-enterobactin uptake [52,53,64]. Our finding that adrenaline, noradrenaline, or adrenochrome enhances the abundance of proteins involved in iron homeostasis, in particular IrgA and Feo, a ferrous iron transporter of the inner membrane indicates that beside adrenaline and noradrenaline, adrenochrome also acts as an iron chelator delivering iron to the pathogen. Whether the catecholamine- or adrenochrome-iron complexes directly facilitate iron uptake, or whether they deliver iron to catechol siderophores like vibriobactin, needs to be addressed in the future.

Adrenaline and noradrenaline are suggested to enable interkingdom signaling through bacterial adrenergic receptors like QseBC and QseEF [56,57]. The abundance of components of signaling and translational- and transcriptional control is changed upon exposure of *V. cholerae* to adrenaline, noradrenaline or adrenochrome. A putative LysR regulator and a putative response regulator are altered in abundance upon treatment with adrenaline, noradrenaline or adrenochrome, although we were not able assign them to a known signaling pathway. Regulation of virulence in *V. cholerae* does not seem to be affected by adrenaline, adrenochrome or noradrenaline. Conversion of noradrenaline to 3,4-dihydroxymandleic acid (DHMA) mediates chemotaxis in *E. coli* [65,66]. The conversion relies on the enzymes TynA and FeaB, a monoamine oxidase and aldehyde dehydrogenase and their transcriptional activator FeaR [66], proteins which *V. cholerae* does not harbor. Therefore, metabolic conversion of noradrenaline as observed in *E. coli* is unlikely to occur in *V. cholerae*.

Indole was identified as a signaling molecule in *V. cholerae*, altering iron homeostasis and especially biofilm formation [55]. Since adrenochrome is structurally similar to indole, and since both agents cause an overlapping proteome response in *V. cholerae*, adrenochrome may be sensed in a comparable manner. How adrenochrome, or indole, achieve the down-shift of an important metabolic enzyme such as methionine synthetase needs to be addressed in further studies.

Adrenochrome has not yet been reported to have immune modulation function, but was discussed to play a role in cardiotoxicity [20] or during septic shock [21]. In a septic-shock rat model 200–600 nM adrenochrome were detected in blood plasma after LPS stimulation [21]. On immune-cell level, adrenaline causes inhibition of LPS-triggered TNF-α production via ß-adrenergic receptors [47]. Adrenochrome also diminishes TNF-α production in monocytic THP-1 cells, but to a remarkably lower extent. We hypothesize that the conversion of adrenaline to adrenochrome may work as molecular sensor indicating the $O_2^{\bullet-}$ occurrence and the need for relative enhancement in TNF-α production (Fig. 7). Conversion of adrenaline to adrenochrome depends on $O_2^{\bullet-}$ which is a result of NQR activity of a pathogen, and of oxidative burst by macrophages. In comparison to adrenaline, adrenochrome exerts reduced inhibitory capacities on TNF-α production in the presence of LPS, thus promoting inflammation (Fig. 7).

To conclude, our study describes a route for adrenochrome formation at the pathogen-host interface, and introduces adrenochrome as novel bacterial effector molecule, iron chelator, and immunomodulatory agent.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgement

We thank Thomas Kufer (University of Hohenheim) for discussions and S. Rautenberg and B. Würtz for excellent technical assistance.

Author contributions

C.T. and J.S. conceived the study and interpreted the findings. C.T. performed all NQR related experiments. C.T., K.M. and J.P. performed and analyzed the proteome study. I.S., C.T. and H.M.M. contributed to the identification of adrenochrome by LC-MS. B.M. and C.T. identified adrenochrome during growth of *V. cholerae*. S.S. and V.S. conducted, performed and evaluated the investigations concerning THP-1 cells. The paper was written by C.T. and J.S. with contributions from all authors.

Funding

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to JS (FR 1321/5-1).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dummy.2019.01.002.

References

[1] J. Steuber, G. Vohl, M.S. Casutt, T. Vorburger, K. Diederichs, G. Fritz, Structure of the *V. cholerae* Na^+-pumping NADH:quinone oxidoreductase, Nature 516 (2014) 62–67.

[2] C.C. Häse, N.D. Fedorova, M.Y. Galperin, P.A. Dibrov, Sodium ion cycle in bacterial pathogens. Evidence from cross-genome comparisons, Microbiol Mol Biol Rev 65 (2001) 353–370.

[3] A. Reyes-Prieto, B. Barquera, O. Juárez, Origin and evolution of the sodium -pumping NADH. Ubiquinone oxidoreductase, PLoS One 9 (2014) e96696.

[4] P. Dibrov, E. Dibrov, T.G. Maddaford, M. Kenneth, J. Nelson, C. Resch, G.N. Pierce, Development of a novel rationally designed antibiotic to inhibit a nontraditional bacterial target, Can. J. Physiol. Pharmacol. 95 (2017) 595–603.

[5] C. Toulouse, K. Metesch, J. Pfannstiel, J. Steuber, Metabolic reprogramming of *Vibrio cholerae* impaired in respiratory NADH oxidation is accompanied with increased copper sensitivity, J. Bacteriol. 200 (2018) e00761-17, , https://doi.org/10.1128/JB.00761-17.

[6] K. Tuz, K.G. Mezic, T. Xu, B. Barquera, O. Juárez, The kinetic reaction mechanism of the *Vibrio cholerae* sodium-dependent NADH dehydrogenase, J. Biol. Chem. 290 (2015) 20009–20021.

[7] M.I. Verkhovsky, A.V. Bogachev, Sodium-translocating NADH:quinone oxidoreductase as a redox-driven ion pump, Biochim. Biophys. Acta 1797 (2010) 738–746.

[8] J. Steuber, G. Vohl, V. Muras, C. Toulouse, B. Claussen, T. Vorburger, G. Fritz, The structure of Na^+ −translocating of NADH ubiquinone oxidoreductase of *Vibrio cholerae*: implications on coupling between electron transfer and Na^+ transport, Biol. Chem. 396 (2015) 1015–1030.

[9] P.C. Lin, K. Türk, C.C. Häse, G. Fritz, J. Steuber, Quinone reduction by the Na^+-translocating NADH dehydrogenase promotes extracellular superoxide production in *Vibrio cholerae*, J. Bacteriol. 189 (2007) 3902–3908.

[10] V. Muras, P. Dogaru-Kinn, Y. Minato, C.C. Häse, J. Steuber, The Na^+ −translocating NADH. Quinone oxidoreductase enhances oxidative stress in the cytoplasm of *Vibrio cholerae*, J. Bacteriol. 198 (2016) 2307–2317.

[11] P.P. Freestone, R.D. Haigh, M. Lyte, Specificity of catecholamine-induced growth in *Escherichia coli* O157. H7, *Salmonella enterica* and *Yersinia enterocolitica*, FEMS Microbiol. Lett. 269 (2007) 221–228.

[12] H. Neuman, J.W. Debelius, R. Knight, O. Koren, Microbial endocrinology. The interplay between the microbiota and the endocrine system, FEMS Microbiol. Rev. 39 (2015) 509–521.

[13] S. Sandrini, M. Aldriwesh, M. Alruways, P. Freestone, Microbial endocrinology. Host-bacteria communication within the gut microbiome, J. Endocrinol. 225 (2015) R21–R34.

[14] W. Bors, C. Michel, M. Saran, E. Lengfelder, The involvement of oxygen radicals during the autoxidation of adrenalin, Biochim. Biophys. Acta 540 (1978) 162–172.

[15] I.C. Szigyártó, L. Szabó, L.I. Simándi, Kinetic studies on the manganese(II) complex catalyzed oxidation of epinephrine, J. Mol. Catal. A Chem. 372 (2013) 66–71.

[16] A. Bindoli, M.P. Rigobello, D.J. Deeble, Biochemical and toxicological properties of the oxidation products of catecholamines, Free Radic. Biol. Med. 13 (1992) 391–405.

[17] F. Remiao, N. Milhazes, F. Borges, F. Carvalho, M.L. Bastos, F. Lemos-Amado, P. Domingues, A. Ferrer-Correia, Synthesis and analysis of aminochromes by HPLC-photodiode array. Adrenochrome evaluation in rat blood, Biomed. Chromatogr. 17 (2003) 6–13.

[18] H.P. Misra, I. Fridovich, The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase, J. Biol. Chem. 247 (1972) 3170–3175.

[19] R. Alhasan, D. Njus, The epinephrine assay for superoxide. Why dopamine does not work, Anal. Biochem. 381 (2008) 142–147.

[20] G.S. Behonick, M.J. Novak, E.W. Nealley, S.I. Baskin, Toxicology update. The cardiotoxicity of the oxidative stress metabolites of catecholamines (aminochromes), J. Appl. Toxicol. 21 (Suppl. 1) (2001) S15–S22.

[21] H. Macarthur, T.C. Westfall, D.P. Riley, T.P. Misko, D. Salvemini, Inactivation of catecholamines by superoxide gives new insights on the pathogenesis of septic shock, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 9753–9758.

[22] D.T. Hughes, V. Sperandio, Inter-kingdom signalling. Communication between bacteria and their hosts, Nat. Rev. Microbiol. 6 (2008) 111–120.

[23] K. Papenfort, B.L. Bassler, Quorum sensing signal-response systems in Gram-negative bacteria, Nat. Rev. Microbiol. 14 (2016) 576–588.

[24] Y. Fu, B.T. Ho, J.J. Mekalanos, Tracking *Vibrio cholerae* cell-cell interactions during infection reveals bacterial population dynamics within intestinal microenvironments, Cell Host Microbe 23 (2018) 274–281 e2.

[25] P. Halang, C. Toulouse, B. Geissel, B. Michel, B. Flauger, M. Müller, R.T. Voegele, V. Stefanski, J. Steuber, Response of *Vibrio cholerae* to the catecholamine hormones epinephrine and norepinephrine, J. Bacteriol. 197 (2015) 3769–3778.

[26] J.J. Mekalanos, D.J. Swartz, G.D. Pearson, N. Harford, F. Groyne, M. de Wilde, Cholera toxin genes nucleotide sequence, deletion analysis and vaccine development, Nature 306 (1983) 551–557.

[27] B. Barquera, P. Hellwig, W. Zhou, J.E. Morgan, C.C. Häse, K.K. Gosink, M. Nilges, P.J. Bruesehoff, A. Roth, C.R.D. Lancaster, R.B. Gennis, Purification and characterization of the recombinant Na^+-translocating NADH:quinone oxidoreductase from *Vibrio cholerae*, Biochemistry 41 (2002) 3781–3789.

[28] M. Tao, G. Fritz, J. Steuber, The Na^+-translocating NADH. quinone oxidoreductase (Na^+-NQR) from *Vibrio cholerae* enhances insertion of FeS in overproduced NqrF subunit, J. Inorg. Biochem. 102 (2008) 1366–1372.

[29] M. Tao, K. Turk, J. Diez, M.G. Grutter, G. Fritz, J. Steuber, Crystallization of the NADH-oxidizing domain of the Na^+-translocating NADH:quinone oxidoreductase from *Vibrio cholerae*, Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 62 (2006) 110–112.

[30] M.S. Casutt, S. Wendelspiess, J. Steuber, G. Fritz, Crystallization of the Na^+-translocating NADH:quinone oxidoreductase from *Vibrio cholerae*, Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 66 (2010) 1677–1679.

[31] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.

[32] S. Green, A. Mazur, E. Shorr, Mechanism of the catalytic oxidation of adrenaline by ferritin, J. Biol. Chem. 220 (1956) 237–255.

[33] The UniProt Consortium, UniProt. The universal protein knowledgebase, Nucleic Acids Res. 45 (2017) D158–D169.

[34] M. Kanehisa, S. Goto, KEGG. Kyoto encyclopedia of genes and genomes, Nucleic Acids Res. 28 (2000) 27–30.

[35] M. Kanehisa, Y. Sato, M. Kawashima, M. Furumichi, M. Tanabe, KEGG as a reference resource for gene and protein annotation, Nucleic Acids Res. 44 (2016) D457–D462.

[36] M. Kanehisa, M. Furumichi, M. Tanabe, Y. Sato, K. Morishima, KEGG. New perspectives on genomes, pathways, diseases and drugs, Nucleic Acids Res. 45 (2017) D353–D361.

[37] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403–410.

[38] M. Johnson, I. Zaretskaya, Y. Raytselis, Y. Merezhuk, S. McGinnis, T.L. Madden, NCBI BLAST. A better web interface, Nucleic Acids Res. 36 (2008) W5–W9.

[39] J.A. Vizcaino, R.G. Cote, A. Csordas, J.A. Dianes, A. Fabregat, J.M. Foster, J. Griss, E. Alpi, M. Birim, J. Contell, G. O'Kelly, A. Schoenegger, D. Ovelleiro, Y. Perez-Riverol, F. Reisinger, D. Rios, R. Wang, H. Hermjakob, The PRoteomics IDEntifications (PRIDE) database and associated tools. status in 2013, Nucleic Acids Research 41 (2013) D1063–9.

[40] J. Steuber, W. Krebs, P. Dimroth, The Na^+-translocating NADH. Ubiquinone oxidoreductase from *Vibrio alginolyticus*–redox states of the FAD prosthetic group and mechanism of ag^+ inhibition, Eur. J. Biochem. 249 (1997) 770–776.

[41] A. Bindoli, D.J. Deeble, M.P. Rigobello, L. Galzigna, Direct and respiratory chain-mediated redox cycling of adrenochrome, Biochim. Biophys. Acta 1016 (1990) 349–356.

[42] M.L. Genova, N.M. Abd-Elsalam, S.M. Mahdy el, A. Bernacchia, M. Lucarini, G.F. Pedulli, G. Lenaz, Redox cycling of adrenaline and adrenochrome catalysed by mitochondrial complex I, Arch. Biochem. Biophys. 447 (2006) 167–173.

[43] C. Toulouse, B. Claussen, V. Muras, G. Fritz, J. Steuber, Strong pH dependence of coupling efficiency of the Na^+ − translocating NADH:quinone oxidoreductase (Na^+-NQR) of *Vibrio cholerae*, Biol. Chem. 398 (2017) 251–260.

[44] X.D. Pfenninger-Li, S.P. Albracht, R. van Belzen, P. Dimroth, NADH:quinone oxidoreductase of *Vibrio alginolyticus*: purification, properties, and reconstitution of the Na^+ pump, Biochemistry 35 (1996) 6233–6242.

[45] M.M. Kushnir, F.M. Urry, E.L. Frank, W.L. Roberts, B. Shushan, Analysis of catecholamines in urine by positive-ion electrospray tandem mass spectrometry, Clin. Chem. 48 (2002) 323–331.

[46] F. Lemos-Amado, P. Domingues, A. Ferrer-Correia, F. Remiao, N. Milhazes, F. Borges, F.D. Carvalho, M.L. Bastos, Electrospray tandem mass spectrometry of aminochromes, Rapid Commun. Mass Spectrom. 15 (2001) 2466–2471.

[47] A. Severn, N.T. Rapson, C.A. Hunter, F.Y. Liew, Regulation of tumor necrosis factor production by adrenaline and beta-adrenergic agonists, J. Immunol. 148 (1992) 3441–3445.

[48] L.A. Grisanti, J. Evanson, E. Marchus, H. Jorissen, A.P. Woster, W. DeKrey, E.R. Sauter, C.K. Combs, J.E. Porter, Pro-inflammatory responses in human monocytes are beta1-adrenergic receptor subtype dependent, Mol. Immunol. 47 (2010) 1244–1254.

[49] P.P. Freestone, M. Lyte, C.P. Neal, A.F. Maggs, R.D. Haigh, P.H. Williams, The mammalian neuroendocrine hormone norepinephrine supplies iron for bacterial growth in the presence of transferrin or lactoferrin, J. Bacteriol. 182 (2000) 6091–6098.

[50] P.P. Freestone, R.D. Haigh, P.H. Williams, M. Lyte, Involvement of enterobactin in norepinephrine-mediated iron supply from transferrin to enterohaemorrhagic *Escherichia coli*, FEMS Microbiol. Lett. 222 (2003) 39–43.

[51] P. Haskova, P. Kovarikova, L. Koubkova, A. Vavrova, E. Mackova, T. Simunek, Iron chelation with salicylaldehyde isonicotinoyl hydrazone protects against catecholamine autoxidation and cardiotoxicity, Free Radic. Biol. Med. 50 (2011) 537–549.

[52] A.R. Mey, E.E. Wyckoff, A.G. Oglesby, E. Rab, R.K. Taylor, S.M. Payne, Identification of the *Vibrio cholerae* enterobactin receptors VctA and IrgA. IrgA is not required for virulence, Infect. Immun. 70 (2002) 3419–3426.

[53] E.E. Wyckoff, B.E. Allred, K.N. Raymond, S.M. Payne, Catechol Siderophore transport by *Vibrio cholerae*, J. Bacteriol. 197 (2015) 2840–2849.

[54] L. Sisinni, L. Cendron, G. Favaro, G. Zanotti, *Helicobacter pylori* acidic stress response factor HP1286 is a YceI homolog with new binding specificity, FEBS J. 277 (2010) 1896–1905.

[55] R.S. Mueller, S. Beyhan, S.G. Saini, F.H. Yildiz, D.H. Bartlett, Indole acts as an extracellular cue regulating gene expression in *Vibrio cholerae*, J. Bacteriol. 191 (2009) 3504–3516.

[56] M.B. Clarke, V. Sperandio, Transcriptional autoregulation by quorum sensing *Escherichia coli* regulators B and C (QseBC) in enterohaemorrhagic *E. coli* (*EHEC*), Mol. Microbiol. 58 (2005) 441–455.

[57] M.B. Clarke, D.T. Hughes, C. Zhu, E.C. Boedeker, V. Sperandio, The QseC sensor kinase. A bacterial adrenergic receptor, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 10420–10425.

[58] G. Nagy, Y. Bartha, T. Keresztes, E. Olveti, M. Madléna, Quantitative analysis of

catecholamines in human dental pulp, J. Endod. 26 (2000) 596–598.
[59] M.A. Flierl, D. Rittirsch, B.A. Nadeau, A.J. Chen, J.V. Sarma, F.S. Zetoune, S.R. McGuire, R.P. List, D.E. Day, L.M. Hoesel, H. Gao, N. van Rooijen, M.S. Huber-Lang, R.R. Neubig, P.A. Ward, Phagocyte-derived catecholamines enhance acute inflammatory injury, Nature 449 (2007) 721–725.
[60] Y. Asano, T. Hiramoto, R. Nishino, Y. Aiba, T. Kimura, K. Yoshihara, Y. Koga, N. Sudo, Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice, Am. J. Physiol. Gastrointest. Liver Physiol. 303 (2012) G1288–G1295.
[61] L.S. Brunner, D.S. Suddarth, Brunner & Suddarth's handbook of laboratory and diagnostic tests, Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, Pa, 2010.
[62] C.L. Burton, S.R. Chhabra, S. Swift, T.J. Baldwin, H. Withers, S.J. Hill, P. Williams, The growth response of *Escherichia coli* to neurotransmitters and related catecholamine drugs requires a functional enterobactin biosynthesis and uptake system, Infect. Immun. 70 (2002) 5913–5923.
[63] S.K. Armstrong, T.J. Brickman, R.J. Suhadolc, Involvement of multiple distinct *Bordetella* receptor proteins in the utilization of iron liberated from transferrin by host catecholamine stress hormones, Mol. Microbiol. 84 (2012) 446–462.
[64] S.M. Payne, A.R. Mey, E.E. Wyckoff, *Vibrio* iron transport. Evolutionary adaptation to life in multiple environments, Microbiol. Mol. Biol. Rev. 80 (2016) 69–90.
[65] N. Sule, S. Pasupuleti, N. Kohli, R. Menon, L.J. Dangott, M.D. Manson, A. Jayaraman, The norepinephrine metabolite 3,4-dihydroxymandelic acid is produced by the commensal microbiota and promotes chemotaxis and virulence gene expression in enterohemorrhagic *Escherichia coli*, Infect. Immun. 85 (2017).
[66] S. Pasupuleti, N. Sule, W.B. Cohn, D.S. MacKenzie, A. Jayaraman, M.D. Manson, Chemotaxis of *Escherichia coli* to norepinephrine (NE) requires conversion of NE to 3,4-dihydroxymandelic acid, J. Bacteriol. 196 (2014) 3992–4000.