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Quantification of nitric oxide by high-performance liquid chromatography-fluorometric method in subgenomic hepatitis C virus-replicon expressing Huh7 cells upon treatment with acetylsalicylic acid

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Abstract. As nitric oxide (NO) expression levels are lower in hepatocytes compared with other cell types, it is difficult to quantify this compound via Griess assay. The aim of the present study was to quantify NO concentration in the cell culture medium from a subgenomic hepatitis C virus (HCV)-replicon expressing Huh-7 cell system using a high-performance liquid chromatography (HPLC)-fluorescence detector in the presence or absence of acetylsalicylic acid (ASA) treatment. HCV-replicon cells were incubated with ASA (4 mM) for 24, 48 and 72 h. Thereafter, the medium was collected to measure nitrites (NO_2^-) as an indirect indicator of NO levels using diaminonaphtalene as a derivate agent. NO levels were significantly higher (1.7-fold) in Huh-7 replicon cells treated with ASA (72 h post-treatment) than untreated cells ($P<0.05$); NO inhibitor reduced ~30% the level of NO in Huh-7 replicon cells treated with ASA (48 h post-treatment; $P<0.05$). The findings suggested that the HPLC-fluorescence method provided an accurate and efficient measurement of NO production in Huh-7-HCV-replicon cells culture medium.

Introduction

Nitric oxide (NO) is an endogenous free radical that has previously been challenging to measure within blood samples, due to its reactive nature and short half-life; however, the Griess assay is currently widely applied to measure NO via the quantification of nitrite (NO_2^-); Wen and Paik (1) indirectly measured aliphatic β-nitroalcohols and the Greiss assay has also been used to investigate the NO-dependent egradation pathway of CYP2C22 (2). Jansen *et al* (3) also used the Greiss assay to study the nitric oxide synthase (NOS) active site. As NO reacts with molecular oxygen to generate nitrate (NO_3^-) and NO_2^-, the latter is soluble in water and is easier to detect (4). The NO_2^- is acidified (usually with phosphoric acid) to form nitrous acid to allow diazotization via the nitrosation of sulfanilic acid. Once sulfanilamide is produced, the reaction with N-1-napthylethylenediamine dihydrochloride produces a purple chromophore with an absorbance at 540 nm (5).

The Griess reaction is inexpensive, simple and fast; however, the limit of detection is ~0.5 μM, which restricts the application of this colorimetric method for quantifying micromolar, nanomolar and picomolar levels of NO_2^-, and therefore physiological amounts (6). Furthermore, the media composition in biological systems may alter the measurements of NO due to the presence of other proteins, such as hemoglobin, which is also detected at 540 nm (7). More sophisticated methods have also been used to measure biological NOS. Independently of whether it is a prokaryotic or eukaryotic culture, NO may be classified via direct or indirect methods. In the latter methods, other biological components may be measured in addition to NO_2^- or NO_3^-, such as guanosine monophosphase (cGMP) (8), L-citrulline (9) and nitrosothiols (10).

As NO is a free radical, electron paramagnetic resonance spectroscopy may be used as a direct method to estimate NO levels; however low levels of NO, poor sensitivity and the complexity of the evaluation of the results, reduces the efficacy of this method in biological contexts (11,12).

Fluorescence assays are more precise, indirect methods to measure NO with a sensitivity of 0.6-0.8 nM. This technique involves a reaction between a secondary metabolite of NO and a derivatizing agent, which generates a fluorescent molecule (6).

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This technique has been used in biological samples, including culture with mammalian cells as it allows real-time measurements, ease of use and high sensitivity (13,14).

High-performance liquid chromatography (HPLC) has been used previously to quantify NO (15) due to its high sensitivity (16) and was identified as a potentially more useful method to quantify NO in hepatocyte cell culture, based on the reaction of NO_2 with 2, 3-diaminonaphthalene (DAN) as an indicator of NO formation, under acidic conditions to obtain the fluorescent product 2, 3-naphthotriazole (NAT) (13). DAN has excitation and emission wavelengths of 375 and 415 nm, respectively, and a 10-30 nM detection limit (14).

NO has numerous molecular targets in various biological schemes, for instance in the cardiovascular system, it stimulates cardioprotective signaling pathways (11); whereas in the nervous system it modulates synaptic functions (17), and has implications in learning, memory and visual recognition (18). Through endogenous metabolic pathways, NO is mainly synthesized by three different isoforms of NOS depending on the cell type and stimulus: Neuronal NOS (nNOS or NOS I), endothelial NOS (eNOS or NOS III) and inducible NOS (iNOS or NOS II) (19).

It has previously been demonstrated that acetylsalicylic acid (ASA) exhibits antiviral properties against hepatitis C virus (HCV) (20). Therefore, one possible explication for the antiviral properties of ASA is that it may be mediated by the antioxidant properties of SOD (21). In addition, it has been confirmed in our laboratory that hepatocytes increase NO expression when exposed to higher concentrations of ASA. However, as NO is produced in the picomolar range within hepatocyte cells culture (5), more precise quantification techniques are required.

The aim of the present study was to measure NO in culture medium of subgenomic HCV-replicon expressing Huh-7 cells following treatment with ASA.

Materials and methods

Cell culture and ASA treatment. Human liver Huh-7 cells containing HCV subgenomic replicon from genotype 1b (harboring the subgenomic HCV replicon I389/NS3-30), the original wild-type $pFKI_{389}$-NS3-3' replicon HCV from genotype 1b and the generation of Huh7 HCV replicon hepatoma cells expressing HCV non-structural proteins have been previously described (22) and were kindly donated by Dr Volker Lohmann (University Hospital Heidelberg, Heidelberg, Germany) (23) were cultured in Advanced Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 2% heat-inactivated fetal bovine serum, 1% non-essential amino acids, 100 U penicillin G, and 100 μg streptomycin/ml (all Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO_2. Cells were maintained in culture in the presence of 500 μg/ml G418 (Gibco; Thermo Fisher Scientific, Inc.) selection agent. Huh-7-HCV-replicon cells were plated the previous day, media was replaced and cells were treated with 4 mM ASA at 37˚C for 24, 48 and 72 h (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Alamar Blue (Invitrogen; Thermo Fisher Scientific, Inc.) was used to determine whether there were any cytotoxic effects; none were observed.

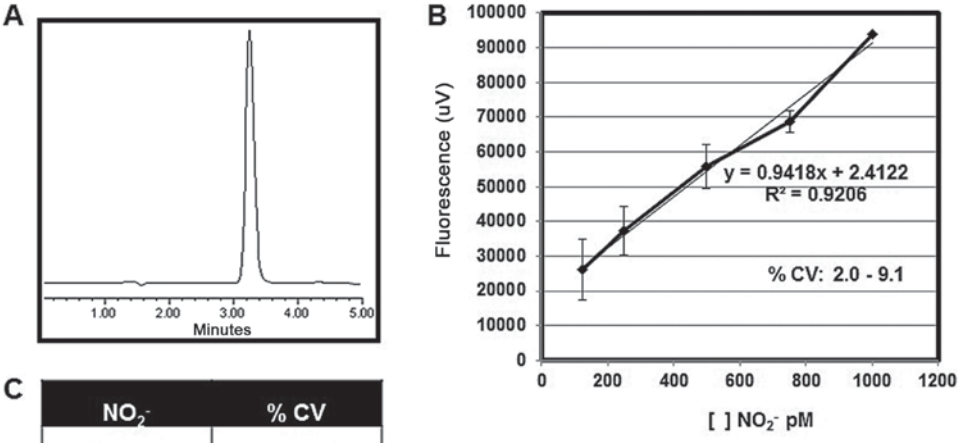
Cell viability assay. Cell viability was assessed in Huh-7 cells using alamar blue in 96-well plates at 37˚C for 24, 48 and 72 h post-incubation. The absorbance was monitored at 570 and 600 nm.

HPLC system. A Waters HPLC system (Waters Corporation, Milford, MA, USA) including a 600E quaternary pump equipped with a column oven, a 717p automatic injector and a 2475 multi-wavelength fluoresce detector was used. The system was controlled by the Empower software version. 2.0 (Waters, Elstree, UK) and the information was processed with the same software. A standard curve was generated with NO_2^- stock. The NO production was quantified in the culture medium through the measurement of nitrites NO_2^- using the HPLC system with a fluorescence detector; λ excitation 375 nm and λ emission 415 nm.

Sample preparation and NO quantification by HPLC analysis. Briefly, 100 μl supplemented culture medium (blank) or 100 μl medium derived from a viral cell culture (45,000 cells) was mixed with 200 μl analytical grade acetonitrile. Samples were homogenized using a vortex for 30 sec and centrifuged at 7,800 x g for 30 min at room temperature. The supernatant was recovered and the solvent was evaporated using a Savant VLP80 Vacuum Pump-Dry Compressor for 3-4 h. Each sample was dehydrated as in the previous step and then reconstituted in 100 μl Milli-Q H_2O. A total of 1 mg 2'-DAN (Sigma-Aldrich; Merck KGaA) was mixed with 1 ml 0.62 M HCl. For each sample (100 μl), 10 μl DAN was added for the conversion to fluorescent NAT. Tubes were incubated at 27˚C for 10 min. Subsequently, 5 μl 2.8 M NaOH was added. These mixtures were directly used for the chromatographic analysis. The mixture was injected into the HPLC equipment using a flow of 1 ml/min and a mobile phase of phosphate:methanol buffer (50:50) under isocratic conditions; 375 and 415 nm were selected as excitation and emission wavelengths, respectively. Each sample was analysed by triplicate.

Mobile phase with 50% of 15 mM sodium phosphate buffer (pH=7.5) and 50% methanol, was pumped through the HPLC system at a flow rate of 1 ml/min. A Symmetry 4.6x150 mm, reversed-phase C18 column and a guard-column packed with C18 were used for the assay, and the inner column was heated to 37˚C. A total of 10 μl of the derivative nitrite solution was injected into the HPLC system. Fluorescence was monitored with an excitation wavelength of 375 nm, emission of 415 nm and a gain of 10.

Measurement of NO. The calibration curve was produced using the culture medium as matrix and the interference by ASA was eliminated by measuring 4 mM ASA without NO_2^- standard (125, 250, 500, 750 and 1,000 pM), and was validated by linear regression. The medium cell-derived Huh-7-HCV replicon-exposed to 4 mM ASA was processed with acetonitrile to remove proteins and retain only the salt deposits. Each sample was incubated at 27˚C for 10 min with 1 mg DAN-derivative agent to yield a fluorescent signal, detected by HPLC with a fluorescence detector; λ excitation 375 nm and λ emission 415 nm. The resulting readings were interpolated in the calibration curve.



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NO_2^-	% CV
125 pM	5.24380673
250 pM	9.14646267
500 pM	6.22127501
750 pM	4.956574
1000 pM	2.00851922

Figure 1. (A) Resulting signal-high-performance liquid chromatography for samples with a retention time of ~3.2 min for NO_2^-. (B) Standard curve of NO_2^-. (C) % CV for calibration curve.

As controls of NO production, arginine (inducer of NO) and inhibitor of NO (L-NIL) were selected to confirm whether the cell model was responsive to ASA stimuli and therefore regulate NO production.

Statistical analysis. Experiments were performed in triplicate and were analyzed using one-way analysis of variance and Dunnett's test. The calibration curves were evaluated by linearity (y= mx+b) and the coefficient of variation was calculated using the following formula: % coefficient of variation (CV)=[(standard deviation/mean) x100] to evaluate the precision between the triplicates. Data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference. The data was analyzed using SPSS version 20 (IBM Corp., Armonk, NY, USA).

Results

Calibration curve linearity and precision. The calibration curve was prepared using different concentrations of NO_2^- standard (125, 250, 500, 750 and 1,000 pM); these concentrations were assayed in triplicate. The curve presented good linearity (R^2=0.9206) and the equation obtained was y=0.9418x+2.4122 (x=NO pM; y=fluorescense unit). The CV range was 2.0-9.1%. In the chromatograms, single peaks were observed with ~3.2 min as retention time (Fig. 1).

Determination of NO in Huh-7-HCV-replicon cells culture medium. Each sample was incubated with DAN (derivative agent) and thereafter analyzed by HPLC. NO levels were significantly higher (1.7 fold) in Huh-7 replicon cells treated with ASA at 72 h post-treatment compared with NO levels from untreated cells (Fig. 2).

To identify non-cytotoxic concentrations of the control solutions in Huh-7 cell line, a cell viability assay with alamar blue was performed (Fig. 3A and B); 1,000 μM was selected for arginine and 250 μM was selected for L-NIL as the non-cytotoxic concentrations in the model (Table I).

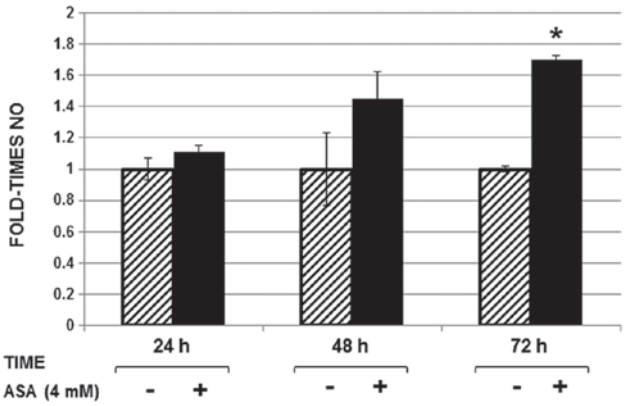

Figure 2. Effect of ASA treatment on NO synthesis in Huh-7-replicon cells. NO production was 1.7-fold higher in Huh-7-replicon cells at 72 h post-treatment, vs. the negative control. *P<0.05 vs. negative control. ASA, acetylsalicylic acid, NO, nitric oxide; CV, coefficient of variation.

At 72 h arginine treatment, NO levels increased (2.3 fold; Fig. 4A), whereas NO levels decreased ~30% at 48 h (P<0.05; Fig. 4B) in Huh-7 replicon cells treated with L-NIL compared with in untreated cells.

Discusion

Hepatocytes produce NO in picomolar levels (6), therefore it is difficult to detect and quantify NO levels within them. In the present study, the HPLC-fluorescence detector method was employed. The aim of the present study was to describe a simple and precise methodology to quantify low concentrations of NO_2^-.

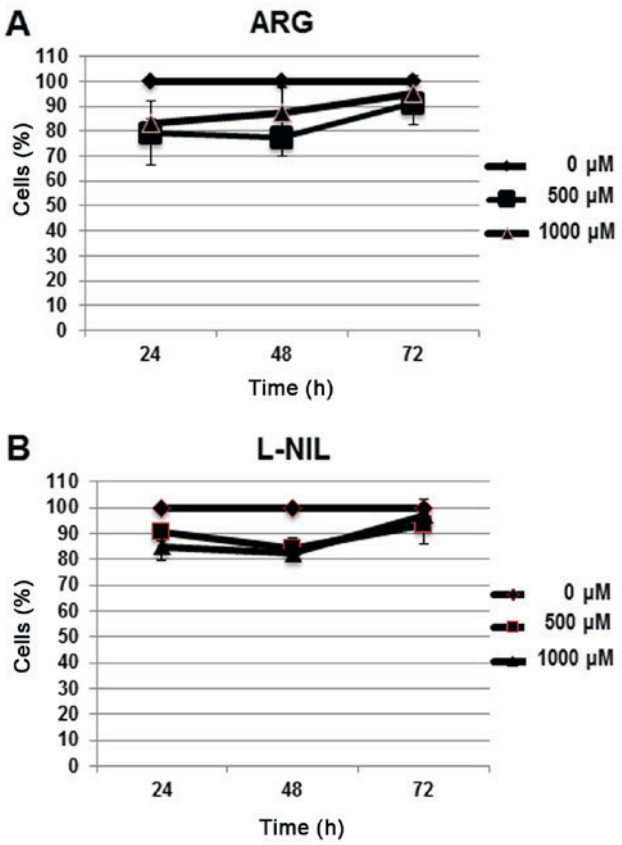

Figure 3. Cell viability assay for ARG and L-NIL. The following non-cytotoxic concentrations were selected: (A) 1,000 μM for ARG and (B) 250 μM for L-NIL. ARG, arginine; L-NIL, inhibitor of nitric oxide.

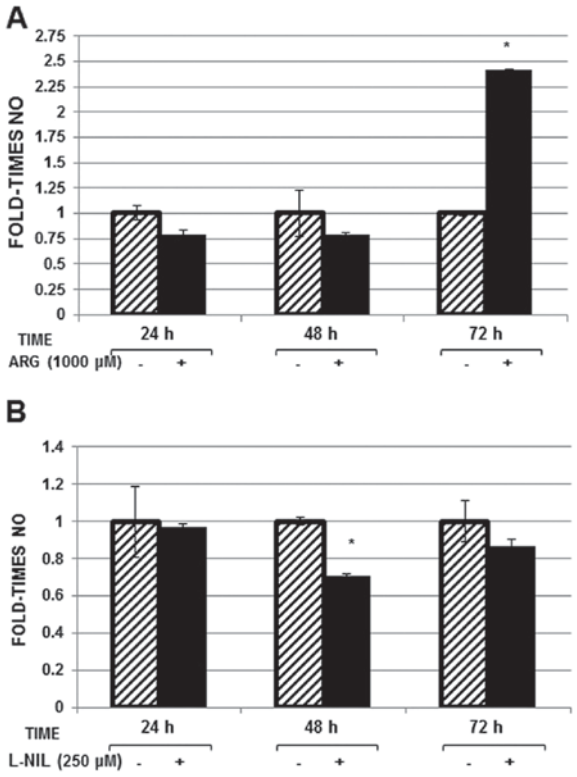

Figure 4. Role of ARG and L-NIL on NO synthesis in Huh-7-replicon cells. (A) ARG increased the amount of NO as did ASA, whereas (B) L-NIL inhibited its production. *P<0.05 vs. negative control. ARG, arginine; L-NIL inhibitor of nitric oxide; NO, nitric oxide.

Table I. Cell viability assay, measuring the percentage of viable cells using alamar blue for 500 and 1,000 μM ARG, and with 100 and 500 μM L-NIL at 24, 48 and 72 h.

A, ARG

Time (h)	0 μM (%)	500 μM (%)	1,000 μM (%)
24	100.00	79.30±12.8	83.00±2.00
48	100.00	77.60±7.70	87.60±10.00
72	100.00	91.00±8.70	95.00±7.20

B, L-NIL

Time (h)	0 μM (%)	100 μM (%)	500 μM (%)
24	100.00	90.33±3.21	85.00±5.29
48	100.00	84.00±4.35	82.33±3.05
72	100.00	93.30±7.23	97.00±6.08

Data are presented as the mean ± standard deviation. ARG, arginine; L-NIL, inhibitor of nitric oxide.

In a previous study performed in our laboratory, ASA increased the activity and expression of superoxide dismutase enzyme (20), which suggested that the antiviral activity of ASA on HCV (22) is mediated, at least in part, by its antioxidant properties. Therefore, the present study aimed to quantify variations in NO levels upon this event under different conditions.

It has been previously reported that iNOS-derived NO has antiviral properties (4). The transcription factors nuclear factor-κB and interferon regulatory factor 1 induce the expression of iNOS to stimulate antiviral activity to inhibit human herpes simplex virus 1 replication in fibroblasts and macrophages (24); however the mechanism by which it exerts the antiviral activity requires further investigation (25). In addition, ASA has been reported to increase NO production in endothelial cells via the NO-cGMP signaling pathway, exhibiting numerous beneficial effects in patients with chronic stable coronary disease (26).

To investigate whether ASA was able to increase the levels of NO in liver cells, Huh-7-HCV replicon cells were exposed to 4 mM ASA for 24, 48 and 72 h. A time-dependent increase by 1.7-fold after 72 h of exposure was observed, confirming the potential of ASA to induce the production of NO. As a control parameter, the levels of NO were measured in cells exposed to 1,000 μM arginine or 250 μM L-NIL (inducer and inhibitor of NO respectively). An increase in the levels of NO was observed in cells exposed to arginine, equivalent to two orders of magnitude, whereas L-NIL reduced NO production and iNOS expression at the transcriptional level. NO was previously quantified using the Griess method (27), but the detection range of the calibration curve using Griess method was 1.56-100 μM, whereas for the HPLC-fluorescence method the range was 125-1,000 pM. Therefore, HPLC may be more useful for the detection of low NO concentrations.

Marzinzig, *et al* (28) previously compared and improved standard methods to determine NO_2^-, NO_3^- and S-nitrosothiol levels in cell culture supernatants. The conventional Griess reaction was modified by replacing sulfanilamide with dapsone (4,4'-diamino-diphenylsulfone) and the NO_2^- levels were measured. The modification, along with ultrafiltration of the samples, resulted in an enhanced sensitivity to measure NO_2^- at 0.2 μM. The detection limit was further improved to 0.02 μM when NO_2^- was identified by DAN.

Li *et al* (29) previously separated NAT on a 5-micron reverse-phase C18 column (inner diameter, 150x4.6 mm) guarded by a 40-μm reverse-phase C18 column (inner diameter, 50x4.6 mm), and eluted with 15 mM sodium phosphate buffer (pH 7.5) containing 50% methanol (flow-rate, 1.3 ml/min). Fluorescence was monitored with excitation at 375 nm and emission at 415 nm with fluorescence intensity of NAT was linear with NO_2^- or NO_3^- concentrations ranging from 12.5-2,000 nM, whereas the curve in the present study was linear between 125 and 1,000 pM.

In the present study, the supernatant media was subjected to evaporation with vacuum and cooling to obtain the salt deposits containing NO_2^-, salts were subsequently reacted with DAN agent for derivatization under acidic conditions (HCl), which reacts with NO_2^- to form a fluorescent molecule, NAT. The reaction was performed for 10 min at 27˚C and the product was stabilized with NaOH. The product of the derivatization was injected (10 μl) into a C18 reverse phase column with a flow of 1 ml/min using as a mobile phase a mixture of 15 mM phosphate buffer pH 7.5 + methanol in a 50:50 ratio under isocratic conditions (the same proportion is always maintained in the mobile phase mixture). Finally, the signal was detected by fluorescence with a 375 nm excitation wavelength and an emission of 415 nm.

The modulation of NO in the presence of ASA in hepatocarcinoma cells with the structural proteins of HCV was quantified in the present study. Therefore, it may be of importance to analyze the effects of NO against the treatment with the complete viral particles.

NO is a soluble and highly labile gas that is released by the conversion of the amino acid L-arginine to L-citrulline. For the synthesis of NO, in addition to L-arginine as substrate, the presence of calmodulin (CaM) and four other cofactors is required: Flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobloptenin (TBH) and nicotinamide adenine dinucleotide phosphate (NADPH) (30). This reaction is catalyzed by the enzyme NOS and can be inhibited by structural derivatives of the amino acids N-mono-methyl-L-arginine and N-nitro-L-arginine methyl esters (31).

When CaM is not bound to the enzyme, the electrons donated by NAPDH do not flow from the reductase domain to the oxygenase domain, but are accepted by cytochrome c and other electron acceptors (32). In the presence of CaM, the electrons donated by NADPH are transported by FAD and by FMN to the heme group. L-arginine is converted to N-hydroxyalanine, then to NO and L-citrulline (33). A limitation of the present study was the fact that it was an *in vitro* investigation. The results of the present study should be validated in samples from patients infected with HCV.

In conclusion, the HPLC-fluorometric method was confirmed to have the precision and linearity to produce confident measures for NO levels in the cellular medium and therefore may be used for routine monitoring of NO variation under various cell media conditions. In the present study, ASA was observed to induce NO production in Huh-7-HCV-replicon cells. These results suggest that NO may be associated with the negative regulation of ASA on HCV, particularly as NO is associated with antiviral properties on certain viruses, including Epstein Barr, Dengue and human immunodeficiency viruses, which supports previous research (33).

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Availability of data and materials

Not applicable.

Authors' contributions

AMRE and CPRI conceived and designed the study. AMRE, CPRI, VTdlC and AGOR analyzed the data. AMRE and CPRI interpreted the results of the experiments. AMRE, CPRI, VTdlC and AGOR drafted the manuscript. AMRE, CPRI and VTdlC performed the experiments. AMRE, CPRI, VTdlC and AGOR prepared the figures. AMRE, CPRI, VTdlC and AGOR edited and revised the manuscript. AMRE, CPRI, VTdlC and AGOR approved the final version of manuscript to be published.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Wen Q and Paik DC: Using the Griess colorimetric nitrite assay for measuring aliphatic β-nitroalcohols. Exp Eye Res 98: 52-57, 2012.
2. Lee CM, Lee BS, Arnold SL, Isoherranen N and Morgan ET: Nitric oxide and interleukin-1β stimulate the proteasome-independent degradation of the retinoic acid hydroxylase CYP2C22 in primary rat hepatocytes. J Pharmacol Exp Ther 348: 141-152, 2014.
3. Jansen Labby K, Li H, Roman LJ, Martásek P, Poulos TL and Silverman RB: Methylated N(ω)-hydroxy-L-arginine analogues as mechanistic probes for the second step of the nitric oxide synthase-catalyzed reaction. Biochemistry 52: 3062-3073, 2013.
4. Mehta DR, Ashkar AA and Mossman KL: The nitric oxide pathway provides innate antiviral protection in conjunction with the type I interferon pathway in fibroblasts. PLoS One 7: e31688, 2012.

5. Gharavi N and El-Kadi AO: Measurement of nitric oxide in murine Hepatoma Hepa1c1c7 cells by reversed phase HPLC with fluorescence detection. J Pharm Pharm Sci 6: 302-307, 2003.
6. Csonka C, Páli T, Bencsik P, Görbe A, Ferdinandy P and Csont T: Measurement of NO in biological samples. Br J Pharmacol 172: 1620-1632, 2015.
7. Hunter RA, Storm WL, Coneski PN and Schoenfisch MH: Inaccuracies of nitric oxide measurement methods in biological media. Anal Chem 85: 1957-1963, 2013.
8. Bartus K, Pigott B and Garthwaite J: Cellular targets of nitric oxide in the hippocampus. PLoS One 8: e57292, 2013.
9. Kolluru GK, Yuan S, Shen X and Kevil CG: H2S regulation of nitric oxide metabolism. Methods Enzymol 554: 271-297, 2015.
10. Hickok JR, Vasudevan D, Antholine WE and Thomas DD: Nitric oxide modifies global histone methylation by inhibiting Jumonji C domain-containing demethylases. J Biol Chem 288: 16004-16015, 2013.
11. Kirca M, Kleinbongard P, Soetkamp D, Heger J, Csonka C, Ferdinandy P and Schulz R: Interaction between connexin 43 and nitric oxide synthase in mice heart mitochondria. J Cell Mol Med 19: 815-825, 2015.
12. Yuan S, Patel RP and Kevil CG: Working with nitric oxide and hydrogen sulfide in biological systems. Am J Physiol Lung Cell Mol Physiol 308: L403-L415, 2015.
13. Ghebremariam YT, Huang NF, Kambhampati S, Volz KS, Joshi GG, Anslyn EV and Cooke JP: Characterization of a fluorescent probe for imaging nitric oxide. J Vasc Res 51: 68-79, 2014.
14. Lowry JL, Brovkovych V, Zhang Y and Skidgel RA: Endothelial nitric-oxide synthase activation generates an inducible nitric-oxide synthase-like output of nitric oxide in inflamed endothelium. J Biol Chem 288: 4174-4193, 2013.
15. Chalupsky K, Kračun D, Kanchev I, Bertram K and Görlach A: Folic acid promotes recycling of tetrahydrobiopterin and protects against hypoxia-induced pulmonary hypertension by recoupling endothelial nitric oxide synthase. Antioxid Redox Signal 23: 1076-1091, 2015.
16. Wu A, Duan T, Tang D, Xu Y, Feng L, Zheng Z, Zhu J, Wang R and Zhu Q: Determination of nitric oxide-derived nitrite and nitrate in biological samples by HPLC coupled to nitrite oxidation. Chromatographia 76: 1649-1655, 2013.
17. Sagi Y, Heiman M, Peterson JD, Musatov S, Scarduzio M, Logan SM, Kaplitt MG, Surmeier DJ, Heintz N and Greengard P: Nitric oxide regulates synaptic transmission between spiny projection neurons. Proc Natl Acad Sci USA 111: 17636-17641, 2014.
18. Tamagnini F, Barker G, Warburton EC, Burattini C, Aicardi G and Bashir ZI: Nitric oxide-dependent long-term depression but not endocannabinoid-mediated long-term potentiation is crucial for visual recognition memory. J Physiol 591: 3963-3979, 2013.
19. Förstermann U and Sessa WC: Nitric oxide synthases: Regulation and function. Eur Heart J 33: 829-837, 837a-837d, 2012.
20. Sánchez-García A, Ríos-Ibarra CP, Rincón-Sánchez AR, Ortiz-López R, Garza-Juárez A, Morlett-Chávez J, Martínez-Rodríguez H and Rivas-Estilla AM: Use of proteomic analysis tools to identify HCV-proteins down-regulated by acetylsalicylic acid. Ann Hepatol 12: 725-732, 2013.
21. Rivas-Estilla AM, Bryan-Marrugo OL, Trujillo-Murillo K, Pérez-Ibave D, Charles-Niño C, Pedroza-Roldan C, Ríos-Ibarra C, Ramírez-Valles E, Ortiz-López R, Islas-Carbajal MC, *et al*: Cu/Zn superoxide dismutase (SOD1) induction is implicated in the antioxidative and antiviral activity of acetylsalicylic acid in HCV-expressing cells. Am J Physiol Gastrointest Liver Physiol 302: G1264-G1273, 2012.
22. Lohmann V, Körner F, Koch JO, Herian U, Theilmann L and Bartenschlager R: Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285: 110-113, 1999.
23. Trujillo-Murillo K, Rincón-Sánchez AR, Martínez-Rodríguez H, Bosques-Padilla F, Ramos-Jiménez J, Barrera-Saldaña HA, Rojkind M and Rivas-Estilla AM: Acetylsalicylic acid inhibits hepatitis C virus RNA and protein expression through cyclooxygenase 2 signaling pathways. Hepatology 47: 1462-1472, 2008.
24. Glatthaar-Saalmüller B, Mair KH and Saalmüller A: Antiviral activity of aspirin against RNA viruses of the respiratory tract-an in vitro study. Influenza Other Respir Viruses 11: 85-92, 2017.
25. Hetzel S, DeMets D, Schneider R, Borzak S, Schneider W, Serebruany V, Schröder H and Hennekens CH: Aspirin increases nitric oxide formation in chronic stable coronary disease. J Cardiovasc Pharmacol Ther 18: 217-221, 2013.
26. Moore TC, Bush KL, Cody L, Brown DM and Petro TM: Control of early Theiler's murine encephalomyelitis virus replication in macrophages by interleukin-6 occurs in conjunction with STAT1 activation and nitric oxide production. J Virol 86: 10841-10851, 2012.
27. Ríos-Ibarra CP, Lozano-Sepulveda S, Muñoz-Espinosa L, Rincón-Sánchez AR, Cordova-Fletes C and Rivas-Estilla AM: Downregulation of inducible nitric oxide synthase (iNOS) expression is implicated in the antiviral activity of acetylsalicylic acid in HCV-expressing cells. Arch Virol 159: 3321-3328, 2014.
28. Marzinzig M, Nussler AK, Stadler J, Marzinzig E, Barthlen W, Nussler NC, Beger HG, Morris SM Jr and Brückner UB: Improved methods to measure end products of nitric oxide in biological fluids: Nitrite, nitrate, and S-nitrosothiols. Nitric Oxide 1: 177-189, 1997.
29. Li H, Meininger CJ and Wu G: Rapid determination of nitrite by reversed-phase high-performance liquid chromatography with fluorescence detection. J Chromatogr B Biomed Sci Appl 746: 199-207, 2000.
30. Morris SM Jr: Enzymes of arginine metabolism. J Nutr 134 (10 Suppl): 2743S-2747S; Discussion 2765S-2767S, 2004.
31. Griffith OW and Stuehr DJ: Nitric oxide synthases: Properties and catalytic mechanism. Annu Rev Physiol 57: 707-736, 1995.
32. Haque MM, Fadlalla MA, Aulak KS, Ghosh A, Durra D and Stuehr DJ: Control of electron transfer and catalysis in neuronal nitric-oxide synthase (nNOS) by a hinge connecting its FMN and FAD-NADPH domains. J Biol Chem 287: 30105-30116, 2012.
33. Takhampunya R, Padmanabhan R and Ubol S: Antiviral action of nitric oxide on dengue virus type 2 replication. J Gen Virol 87: 3003-3011, 2006.