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Valproic acid promotes a decrease in mycobacterial survival by enhancing nitric oxide production in macrophages stimulated with IFN-γ

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ABSTRACT

Tuberculosis is one of the leading causes of mortality worldwide, it is caused by *Mycobacterium tuberculosis* (Mtb), a bacteria that employs several strategies to evade the host immune response. For instance, Mtb interferes with the overexpression of class II transactivator (CIITA) in macrophages exposed to IFN-γ by inhibiting histone acetylation at its promoter, which can be reverted by the histone deacetylase inhibitor (HDACi) sodium butyrate. In this work, we evaluated whether a different HDACi, valproic acid (VPA), could revert the inhibition of gene expression induced by Mtb. J774 macrophages treated with VPA and IFN-γ unexpectedly induced a higher expression of the inducible nitric oxide synthase and a higher production of nitric oxide when exposed to the 19 kDa lipoprotein of Mtb or the whole bacteria. However, VPA was unable to revert the inhibition of CIITA expression induced by the 19 kDa lipoprotein of Mtb. Finally, macrophages infected with Mtb and treated with VPA and IFN-γ showed a significant reduction in intracellular bacteria. Our findings suggest a new therapeutic potential of VPA for the treatment of tuberculosis.

1. Introduction

Tuberculosis is one of the leading causes of mortality worldwide, it is caused by the bacteria *Mycobacterium tuberculosis* (Mtb). In order to control this disease new strategies are emerging to improve the diagnostic, treatment and prevention of Mtb infection [1]. The immune response against Mtb has a dual role in containing the infection. During the early phase of infection macrophages are infected by Mtb, and the bacteria multiplies intracellularly, leading to macrophage death and dissemination of the bacteria. The induction of a T cell response, where $CD4^+$ T cells produce abundant IFN-γ, leads to the activation of microbicidal mechanisms in macrophages that control the intracellular multiplication of the bacteria, like the production of nitric oxide (NO) mediated by the inducible nitric oxide synthase (iNOS) which is regulated at transcriptional level. However, this response does not lead to the elimination of the bacteria, but to the formation of a Mtb containment structure known as granuloma [2].

Mtb is one of the most successful pathogens that infects the human population because of the many mechanisms that it employs in order to override the host immune response. One of these mechanisms is through regulating host gene expression. In this line, previous studies showed that Mtb is able to inhibit the macrophage gene expression induced by IFN-γ, like CD64, HLA-DR and class II transactivator (CIITA) [3]. One of the mechanisms responsible for this altered gene expression was associated to histone hypoacetylation at the CIITA promoter. Interestingly, CIITA expression could be partially restored with the histone deacetylation inhibitor (HDACi) sodium butyrate [4].

Valproic acid (VPA) is an HDACi that is employed in cancer treatment because of its ability to induce the expression of genes that were epigenetically repressed during tumorigenesis and that regulate different processes like cell cycle arrest, apoptosis, angiogenesis, metastasis, cell differentiation, cell senescence and immune recognition [5].

Abbreviations: Mycobacterium tuberculosis, Mtb; histone deacetylase inhibitor, HDACi; valproic acid, VPA; class II transactivator, CIITA; inducible nitric oxide synthase, iNOS; nitric oxide, NO; multiplicity of infection, MOI; hours post-infection, hpi; colony forming units, CFU

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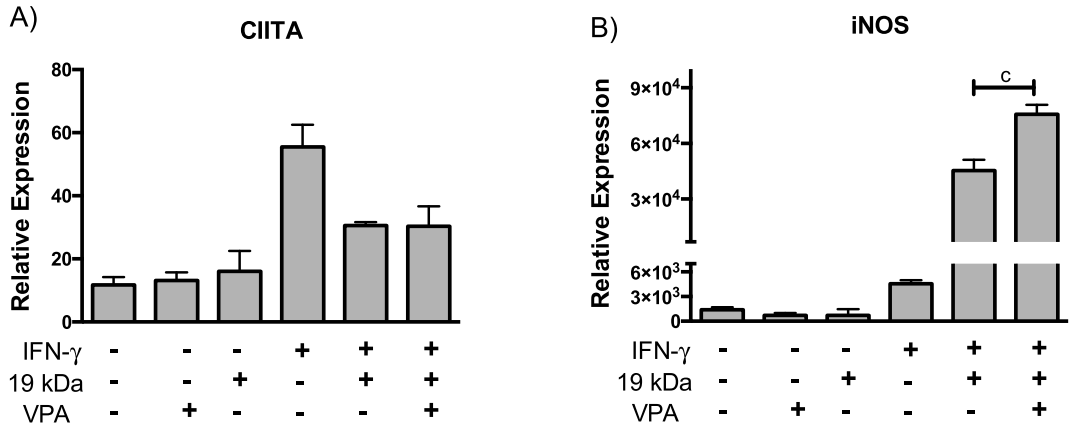

Fig. 1. Valproic acid induces an increased mRNA expression of iNOS in J774 macrophages stimulated with the 19 kDa Mtb lipoprotein and IFN-γ. J774 macrophages were cultured in the presence or absence of 1 mM VPA and stimulated for 18 h with IFN-γ and/or 19 KDa Mtb lipoprotein. mRNA for (A) CIITA and (B) iNOS was evaluated by q-PCR. Data are presented as the mean ± SD of three experiments. c, $p < 0.01$.

In view of these facts, we tested whether VPA was able to revert the gene expression inhibition induced by Mtb and whether intracellular mycobacterial viability was affected. Interestingly, we noticed that VPA was unable to revert the inhibition of CIITA induced by Mtb, but favored a higher expression of iNOS and NO in J774 macrophages stimulated with IFN-γ, which was related with a reduced Mtb intracellular load. These results suggest a potential therapeutic interest of VPA in tuberculosis.

2. Methods

2.1. Cell culture

Mycobacterium tuberculosis H37Rv was cultured following standard procedures [6]. The J774A.1 murine macrophages cell line (ATCC, USA) was cultured in RPMI medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37 °C and 5% CO_2. To stimulate macrophages, we used a protocol previously described, with some modifications [4]. Briefly, macrophages were incubated for 30 min with 1 mM sodium valproate (Sigma, USA), and stimulated for 18 h with the Mtb 19 kDa lipoprotein (19 kDa) (donated by Dr. Mahavir Singh, Lionex GmbH, Germany), or infected with Mtb strain H37Rv (MOI 20), and stimulated with 10 ng/ml IFN-γ (Peprotech, USA).

2.2. Quantitative real-time PCR

To measure CIITA and iNOS mRNA a quantitative real-time PCR was performed as previously described [7]. Briefly, total RNA was obtained with TRIzol Reagent (Invitrogen, USA) from macrophages cultured in different conditions. Reverse transcription of RNA was carried out using MMLV Reverse Transcriptase (Invitrogen, USA). 25 ng cDNA were amplified by q-RT PCR in a LightCycler 1.5 Roche, using primers and probes for CIITA, iNOS and GAPDH (Taqman Gene Expression Assay, Applied Biosystems). Cycle threshold (C_T) values for CIITA and iNOS were normalized to GAPDH using the following equation: $1.8^{\wedge}(C_T$ GAPDH-C_T gene) X 10,000.

2.3. Evaluation of NO production

NO production was evaluated by measuring the levels of its non-volatile breakdown product NO_2^- in cell supernatants with the Griess Reagent System (Promega, USA).

2.4. Evaluation of intracellular Mycobacterium tuberculosis

Macrophages were incubated as previously described, but Mtb infection was performed for 2 h. Extracellular bacteria were eliminated by extensive washing with Hanks Balanced Salt Solution (Gibco, USA) and treated for 2 h with 200 μg/ml amikacin to eliminate extracellular bacteria. After washing to remove the antibiotic, cells were stimulated for 4 or 24 h post-infection (hpi) with 10 ng/ml IFN-γ. Finally, cells were lysed and colony-forming units (CFU) were determined by serial dilution and seeding in Middelbrok-7H10 agar (BD-Difco, USA).

Data is presented as the mean ± SD of three experiments. Statistical differences were determined by one-way ANOVA with Newman-Keuls post-test method (GraphPad Prism Software V6, USA). A p-value < 0.05 was considered significant.

3. Results

Previous reports showed that Mtb, or its products that activate TLR-2, are able to recruit HDAC and inhibit IFN-γ induced expression of CIITA through hypoacetylation of histones associated with its promoter region. This event can be partially reverted by the HDACi sodium butyrate [4]. First, we asked whether this effect could be mimicked by a different HDACi, the short chain fatty acid valproic acid. We noticed that VPA was unable to restore IFN-γ-induced CIITA expression in the presence of the 19 kDa lipoprotein (Fig. 1A); however, we noticed that macrophages cultured with VPA, 19 kDa lipoprotein and IFN-γ had higher levels of iNOS expression, when compared to macrophages cultured with 19 kDa and IFN-γ (Fig. 1B).

Because iNOS is essential for the production of reactive nitrogen species, we assessed whether VPA induced an increased production of NO in macrophages stimulated with IFN-γ and either with 19 kDa or whole Mtb. We observed that macrophages cultured with VPA, 19 kDa lipoprotein and IFN-γ produced higher levels of NO in relation to macrophages that were only stimulated with the 19 kDa and IFN-γ (Fig. 2 A). Furthermore, this effect was more pronounced when macrophages were stimulated with the whole bacteria, instead of the 19 kDa (Fig. 2B).

NO production mediated by iNOS plays a crucial role in controlling intracellular growth of Mtb in macrophages [8], so we decided to evaluate whether VPA had an effect in the elimination of intracellular Mtb. At 24 hpi infected macrophages that were cultured with VPA and IFN-γ showed a significant decrease in the amount of intracellular Mtb, compared with macrophages that were cultured only with IFN-γ, which also showed a reduced number of intracellular bacteria compared with untreated macrophages (Fig. 3A). This difference in bacterial load between macrophages stimulated with IFN-γ and those stimulated with IFN-γ and VPA was lost after 48 hpi. Because previous reports showed that VPA altered the phagocytosis of Gram-positive and Gram-negative bacteria [9], we tested if VPA affected Mtb phagocytosis by

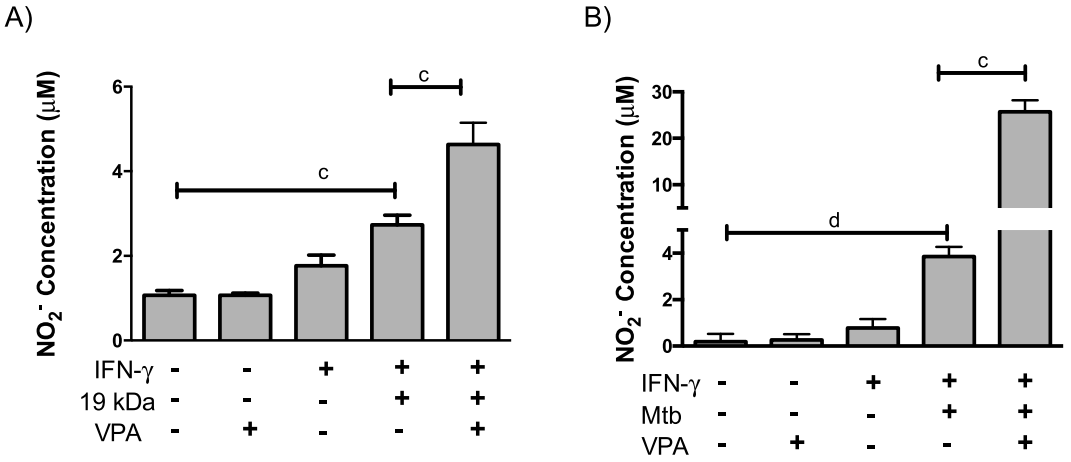

Fig. 2. Valproic acid induces an increased production of NO in J774 macrophages stimulated with Mtb or the 19 kDa Mtb lipoprotein and IFN-γ. J774 macrophages were cultured in the presence or absence of 1 mM VPA and stimulated for 18 h with either the 19 KDa Mtb lipoprotein (A) or whole Mtb (B) and with 10 ng/ml of IFN-γ. NO_2^- was evaluated in cell supernatants with the Griess Reagent System. Data are presented as the mean ± SD of three experiments. c, $p < 0.01$; d, $p < 0.05$.

macrophages. We observed that macrophages exposed to VPA showed a similar number of intracellular Mtb compared with macrophages that were not exposed to VPA after 2 h of infection (Fig. 3B). Furthermore, at 24 hpi macrophages that were treated only with VPA did not show a different number of intracellular bacteria compared with untreated macrophages (Fig. 3B). Taken as a whole, our results show that VPA, an iHDAC, favors the IFN-γ-induced production of iNOS and, in consequence, in the release of NO during Mtb infection, which helps to eliminate intracellular bacteria *in vitro*.

4. Discussion

Although Mtb can evade the immune response through regulation of the host gene expression, these alterations can be modified by the employment of drugs with the ability to reverse these changes. Examples of these drugs are iHDACs, like sodium butyrate, which can revert the down-modulation of CIITA [4]. Interestingly VPA, a different iHDAC, was unable to revert the inhibition of CIITA, probably due to differences in HDAC affinity and specificity between sodium butyrate and VPA [10]. In addition, we now show that VPA facilitates the production of NO by promoting the expression of iNOS, which is one important microbicidal mechanism for the intracellular containment of Mtb. Previous reports showed that VPA was able to reduce the expression of iNOS after stimulation with either IFN-γ or LPS [11,12]; this discrepancy with our results could be partially explained by the differences in the source of macrophages employed in these studies. Furthermore, it is known that Mtb synergizes with IFN-γ for the expression of several genes, including iNOS. Moreover, macrophages stimulated simultaneously with Mtb and IFN-γ express 457 genes that are not induced by Mtb or IFN-γ alone, reflecting a difference in cell signaling [13]. Interestingly, sodium butyrate is able to increase the expression of iNOS and the production of NO in endothelial cells stimulated simultaneously with IFN-γ and LPS [14]. We hypothesize that VPA is able to differentially modulate cell signaling, leading to an increased expression of iNOS, but further work is needed to clarify this issue.

VPA is also known to participate in the regulation of the intracellular growth of Mtb through the induction of autophagy; for instance, previous studies showed that VPA was able to induce autophagy in human, murine alveolar and RAW264.7 macrophages infected with Mtb [15,16]. Furthermore, VPA enhanced the effect of isoniazid and rifampicin in the control of intracellular Mtb survival [17]. In this line, our results show a new mechanism induced by VPA, in addition to its ability to induce autophagy; VPA is also able to favor the expression of iNOS and NO, which are crucial to control intracellular Mtb growth. Our *in vitro* experiments show a potential therapeutic effect of VPA during Mtb infection, but further work is needed to test whether this

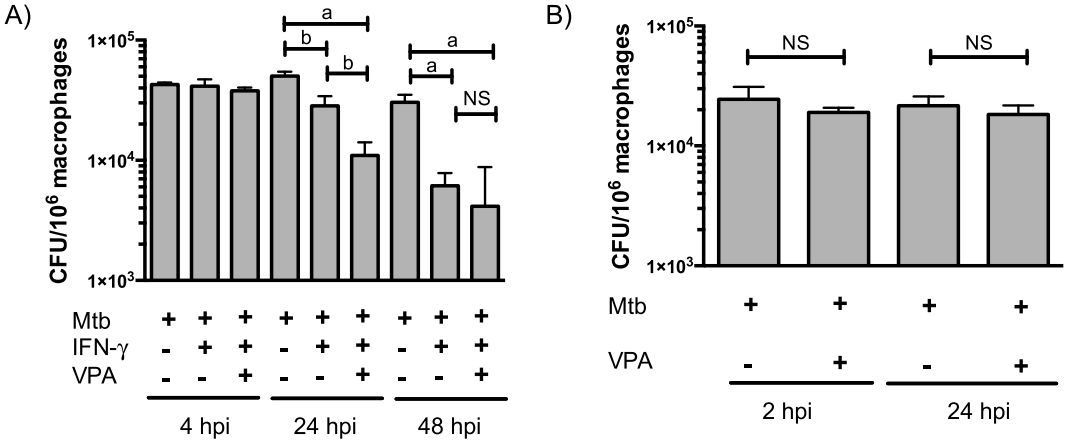

Fig. 3. Valproic acid induces a decrease in viable *Mycobacterium tuberculosis* in J774 macrophages stimulated with IFN-γ. (A) Macrophages were infected for 2 h in the presence or absence of VPA and stimulated for 4, 24 or 48 hpi with 10 ng/ml IFN-γ. Intracellular bacteria were evaluated. (B) J774 macrophages were infected for 2 or 24 h with Mtb in the presence or absence of VPA, and intracellular bacteria were evaluated. Data are presented as the mean ± SD of three experiments. NS: Not significant; a, $p < 0.0001$; b, $p < 0.001$.

effect is relevant in a mouse model of infection and in human tuberculosis.

Conflict of interests

None declared.

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References

[1] WHO. Global tuberculosis report 2017 Geneva, Switzerland: WHO; 2017 Available from: http://www.who.int/tb/publications/global_report/gtbr2017_main_text.pdf.
[2] O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MP. The immune response in tuberculosis. Annu Rev Immunol 2013;31:475–527.
[3] Kincaid EZ, Ernst JD. Mycobacterium tuberculosis exerts gene-selective inhibition of transcriptional responses to IFN-gamma without inhibiting STAT1 function. J Immunol 2003;171(4):2042–9.
[4] Pennini ME, Pai RK, Schultz DC, Boom WH, Harding CV. Mycobacterium tuberculosis 19-kDa lipoprotein inhibits IFN-gamma-induced chromatin remodeling of MHC2TA by TLR2 and MAPK signaling. J Immunol 2006;176(7):4323–30.
[5] Duenas-Gonzalez A, Candelaria M, Perez-Plascencia C, Perez-Cardenas E, de la Cruz-Hernandez E, Herrera LA. Valproic acid as epigenetic cancer drug: preclinical, clinical and transcriptional effects on solid tumors. Cancer Treat Rev 2008;34(3):206–22.
[6] Campillo-Navarro M, Leyva-Paredes K, Donis-Maturano L, Rodriguez-Lopez GM, Soria-Castro R, Garcia-Perez BE, et al. Mycobacterium tuberculosis catalase inhibits the formation of mast cell extracellular traps. Front Immunol 2018;9:1161.
[7] Reyes-Martinez JE, Nieto-Patlan E, Nieto-Patlan A, Gonzaga-Bernachi J, Santos-Mendoza T, Serafin-Lopez J, et al. Differential activation of dendritic cells by Mycobacterium tuberculosis Beijing genotype. Immunol Invest 2014;43(5):436–46.
[8] Chan J, Xing Y, Magliozzo RS, Bloom BR. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. J Exp Med 1992;175(4):1111–22.
[9] Mombelli M, Lugrin J, Rubino I, Chanson AL, Giddey M, Calandra T, et al. Histone deacetylase inhibitors impair antibacterial defenses of macrophages. J Infect Dis 2011;204(9):1367–74.
[10] Santini V, Gozzini A, Ferrari G. Histone deacetylase inhibitors: molecular and biological activity as a premise to clinical application. Curr Drug Metab 2007;8(4):383–93.
[11] Guo L, Guo H, Gao C, Mi Z, Russell WB, Kuo PC. Stat1 acetylation inhibits inducible nitric oxide synthase expression in interferon-gamma-treated RAW264.7 murine macrophages. Surgery 2007;142(2):156–62.
[12] Serrat N, Sebastian C, Pereira-Lopes S, Valverde-Estrella L, Lloberas J, Celada A. The response of secondary genes to lipopolysaccharides in macrophages depends on histone deacetylase and phosphorylation of C/EBPbeta. J Immunol 2014;192(1):418–26.
[13] Ehrt S, Schnappinger D, Bekiranov S, Drenkow J, Shi S, Gingeras TR, et al. Reprogramming of the macrophage transcriptome in response to interferon-gamma and Mycobacterium tuberculosis: signaling roles of nitric oxide synthase-2 and phagocyte oxidase. J Exp Med 2001;194(8):1123–40.
[14] Morikawa A, Sugiyama T, Koide N, Mori I, Mu MM, Yoshida T, et al. Butyrate enhances the production of nitric oxide in mouse vascular endothelial cells in response to gamma interferon. J Endotoxin Res 2004;10(1):32–8.
[15] Schiebler M, Brown K, Hegyi K, Newton SM, Renna M, Hepburn L, et al. Functional drug screening reveals anticonvulsants as enhancers of mTOR-independent autophagic killing of Mycobacterium tuberculosis through inositol depletion. EMBO Mol Med 2015;7(2):127–39.
[16] Juarez E, Carranza C, Sanchez G, Gonzalez M, Chavez J, Sarabia C, et al. Loperamide restricts intracellular growth of Mycobacterium tuberculosis in lung macrophages. Am J Respir Cell Mol Biol 2016;55(6):837–47.
[17] Rao M, Valentini D, Zumla A, Maeurer M. Evaluation of the efficacy of valproic acid and suberoylanilide hydroxamic acid (vorinostat) in enhancing the effects of first-line tuberculosis drugs against intracellular Mycobacterium tuberculosis. Int J Infect Dis 2018;69:78–84.