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Amplification of the Inflammatory Cellular Redox State by Human Immunodeficiency Virus Type 1-Immunosuppressive Tat and gp160 Proteins

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In the course of our studies on oxidative stress as a component of pathological processes in humans, we showed that microintrusion into cells with microcapillary and ultramicroelectrochemical detection could mimic many types of mechanical intrusion leading to an instant (0.1 s) and high (some femtomoles) burst release of H_2O_2. Specific inhibitors of NADPH enzymes seem to support the assumption that this enzyme is one of the main targets of our experiments. Also, human immunodeficiency virus type 1 (HIV-1) gp160 inhibits the cooperative response of uninfected T cells as well as Tat protein release by infected cells does. In this study, we analyzed in real time, lymphocyte per lymphocyte, the T-cell response following activation in relation to the redox state. We showed that the immunosuppressive effects of HIV-1 Tat and gp160 proteins and oxidative stress are correlated, since the native but not the inactivated Tat and gp160 proteins inhibit the cellular immune response and enhance oxidative stress. These results are consistent with a role of the membrane NADPH oxidase in the cellular response to immune activation.

Survival of all living entities is dependent on the modulation of cell metabolism in response to environmental changes and challenges. In higher organisms such phenomena include a defensive response to "stresses" (injury, viruses, and pathogens). Organisms require a rapidly acting system to detect and combat such potentially life-threatening occurrences. Meanwhile, it is known that human metabolism often is not capable of controlling the damaging effects of reduced and reactive oxygen species (ROS), which contribute to several inflammatory processes, apoptosis, carcinogenesis, aging, and human immunodeficiency virus (HIV) expression (2, 15, 18, 24, 28, 30, 33, 34, 37). Our previous data showed that microintrusion with a microcapillary into a single cell, followed by ultramicroelectrochemical detection (4), could mimic many types of mechanical intrusion (e.g., bacteria, virus internalization, asbestos, and endocytocis) leading to an instant (0.1 s) and high (some femtomoles) burst release of H_2O_2. The effects of specific inhibitors of NADPH oxidase or NADPH oxidase-like enzymes seem to support the assumption that this membranic enzyme may be, at least, one of the main targets in our experiments that involved either human fibroblast cell lines or peripheral blood mononuclear cells (PBMCs) from healthy patients and cells from AIDS patients (3, 5). One of these inhibitors endowed with convenience efficacy (maximum efficiency versus minimum cytotoxicity) is phenylarsine oxide (PAO). It is considered to be a specific inhibitor of the NADPH oxidase in human neutrophils (22) and was shown to inhibit phorbol myristate acetate (PMA)-induced oxygen burst in macrophages and neutrophils, the tumor necrosis factor alpha (TNF-α)-induced activation of NF-κB in ML1-a cells, and cytosolic protein kinase C activity in unstimulated neutrophils (6, 8, 25, 26). In the present study we have investigated the mechanisms displayed by the virus in producing oxidative stress. Indeed, in HIV type 1 (HIV-1)-infected individuals, pathogenic processes may be generated by infected cells, which, following immune activation, induce both premature cytolysis and release of virions (14). However, the low percentage of infected cells in the host cannot per se account for the overall immune impairment (2, 7, 27). HIV-1-induced immune disorders should also involve uninfected cells resulting in a progressive T-cell loss by apoptosis or immunosuppression following immune activation with the possible loss of innate immunity. We, and others, have investigated the roles played by two major HIV-1 components, namely the structural Env gp160 and the regulatory Tat proteins, in HIV-1 pathogenesis (29, 38, 39). We have previously shown that gp160 and Tat proteins exert, in a dose-dependent manner, an antiproliferative effect on normal in vitro-activated PBMCs. It is known that Env gp160 or gp120 is present in the extracellular compartment as a soluble molecule released by infected cells after their lysis or as the external protein on free virions causing CD4 cell anergy of uninfected immune cells (27). Tat is an early protein synthesized by infected cells during HIV-1 replication (9, 40). This protein is known to play a major regulatory role in these cells, activating viral replication and blocking cellular metabolism (38, 40). Furthermore, in acute HIV-1 infection with a high replication rate, Tat is released into the extracellular compartment, and at high concentrations, acting as a true toxin, Tat proteins enter noninfected cells either by recognizing integrine receptors or by cell-to-cell contact (11, 12). Uptake of exogenous Tat may cause metabolic changes in noninfected immune cells, leading to immunosuppression as a result of inhibition of the proliferation of antigen-stimulated T cells (21, 38) or to apoptosis (35).

Previous studies have shown that HIV-1 Tat protein could alter the cellular redox state by potentiating the TNF-induced NF-κB activation (36). Additional data show that both antioxi-

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dants and dipyridamole inhibit HIV-1 gp120-induced free radical-based oxidative damage to human monocytic cells (23).

It has also been reported that, as for many other pathological processes, HIV-1 infection is dependent on both the balance between alteration of cellular metabolism and the body response to the induced environmental changes. Therefore, to counteract the cellular damaging effects of HIV-1 Tat and gp160 proteins it was necessary to develop a technique which would allow rapid detection of deleterious effects. The very sensitive method that we have previously developed (3, 4, 5) can be used successfully to analyze, at the level of an individual lymphocyte, the pathogenic role of a Tat- or gp160-induced oxidative stress on cell physiology by using particular inhibitory drugs to quench the effects induced in this way.

The aim of the present study was twofold. First, we sought to evaluate the early relationship between an increased oxidative stress response and the immunosuppressive effects of HIV-1 Tat and gp160 proteins, which inhibit the normal immune response. For this, the redox response of Tat- or gp160-exposed cells was measured cell per cell, in real time (0.1 to 20 s for the full response). Second, we looked for a possible way of reducing ex vivo the damaging effects of HIV-1 Tat and gp160 proteins by combining microelectrochemical and immunological methods.

MATERIALS AND METHODS

Viral proteins. HIV-1 Tat protein was prepared either by DEAE chromatography followed by gel filtration or by solubilization in 6 M guanidine, HCl-containing buffer followed by chromatography on nickel chelate agarose (NTA; Qiagen, Hilden, Germany). Tat cDNA expression vectors were derived from human T-cell lymphotropic virus type IIIB ($HTLV_{IIIB}$) pCV_1. The biological activity of Tat protein was determined by using the chloramphenicol acetyltransferase (CAT) assay on HeLa cells (23). Inactivated Tat protein (iTat) was obtained by using glutaraldehyde in a procedure similar to that used for the preparation of toxoids from bacterial toxins. Abolition of the biological activity of Tat was ascertained by the CAT assay (23). Recombinant gp160, Nef, and Vpr proteins from HIV-1 LAI were a gift from Institut Mérieux (Lyon, France), and inactivated gp160 (igp160) and heat-inactivated Tat (h-Tat) were prepared by heating native proteins for 5 min at 100°C.

Cells. PBMCs were isolated on Ficoll Hypaque from heparinized blood samples taken from healthy donors.

Cell activation. PBMCs (10^6 cells/ml) were stimulated by superantigen staphyloccal enterotoxin type B (SEB) (1 mg/ml) in RPMI medium containing 10% human serum AB and cultured in 96-well round-bottomed culture plates for 12 to 15 h for the study of oxidative stress or for 3 days for the T-cell proliferation assay.

T-cell proliferation was measured by the addition of 1 μCi of [^{3}H]thymidine per well during the last 18 h of culture time, followed by cell harvesting and the quantification of [^{3}H]thymidine incorporation with a β counter.

H_2O_2 detection by ultramicroelectrodes on single PBMCs. The various methods used to prepare platinized carbon-fiber ultramicroelectrodes and detect ex vivo H_2O_2 production by a single human cell have been previously described (3–5). In the present experiments, platinized (maximum electrodeposition current, −500 nA) carbon fiber ultramicroelectrodes (10 μm diameter) were used (Fig. 1) (4, 5). H_2O_2 detection (a few femtomoles) was carried out by amperometry (applied potential of +600 mV/the reference electrode Ag-AgCl) by one of our methods, which consists of puncturing the cell membrane with a closed-tip micropipette (diameter, <0.5 μm) while the detecting ultramicroelectrode is set at 1 to 5 μm above the membrane puncture. Angles of microelectrode position versus the cell surface, and of micropipette intrusion into the cell, have been precisely defined (45°) and were held constant all through experiments. Since this method requires adherent cells, measurements were performed on cells immobilized in petri dishes in the presence of phosphate-buffered saline (PBS) (3). For this, petri dishes were coated for 1 h with 250 μl of poly-L-lysine (1 mg/ml) (P1524; Sigma) dissolved into PBS. Aliquots of about 20,000 lymphocytes from cell culture were deposited on petri dishes. After 10 min, cells were washed and only adherent cells were further treated. Under well-defined conditions, in order to prevent the cells from being damaged in the absence of growth medium, adherent cells in each petri dish were used for only 20 min. As previously stated (4), the cell response consists essentially in a flux of activated oxygen species resulting in further production of $O_2^{\cdot -}$ derivatives, among which is H_2O_2.

Inhibition of NADPH oxidase activity by PAO. We and others have shown that H_2O_2 production in activated PBMCs was dependent on NADPH oxidase activation (3). Such production is highly increased in HIV-1-infected cells (22, 28). In order to verify whether the exogenous immunosuppressive HIV-1 Tat and gp160 proteins were responsible for the H_2O_2 increase, experiments similar to those described above were carried out with PAO at a concentration capable of inhibiting up to 75 to 85% of the H_2O_2 production (3). SEB-activated cells were incubated for 1 h with 25 μM freshly prepared PAO (the stock solution is prepared daily in 95% ethanol at a final concentration of 0.4 M, and the solution is sonicated for 30 min with a Branson 1210 sonicator). Cells were then washed three times with PBS, and the cell membrane was punctured. Routine controls through all experiments included determination of: (i) cell viability (measured within 1 h for PAO at 1 mM); (ii) cellular adhesion; and (iii) recovery of electrochemical response for H_2O_2 production when fresh cells not incubated with catalase or PAO were immediately analyzed. Samples (at least 30 cells) from the various cell preparations were randomly tested over the ensuing 1 day in order to eliminate any possible "time effect." Data are the mean values of the different measurements. At the end of each set of experiments, the ultramicroelectrodes were retested to assess measurement reliability.

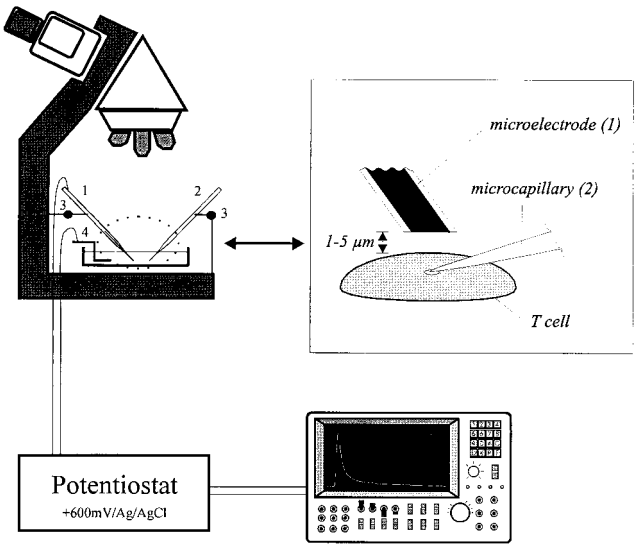

FIG. 1. Schematic representation of the microelectrochemical device for the determination of H_2O_2 production by a single cell in real time. Two three-axis micromanipulator arms (3) are used to set up the ultramicroelectrode (1) and microcapillary tips above the cell surface (2). The cellular response induced by the microcapillary introduction into the lymphocyte is recorded on an amperometer at a fixed potential of +600 mV/(Ag-AgCl) imposed between the ultramicroelectrode and the reference electrode Ag-AgCl (4) by the potentiostat. The angle of the ultramicroelectrode and the microcapillary to the cell is, once and for all, fixed at 45°.

RESULTS

Antiproliferative activity of HIV-1 Tat and gp160 proteins. The proliferative response exhibited by SEB-activated normal PBMCs was markedly reduced when T-cell activation was performed in the presence of HIV-1 Tat or HIV-1 gp160 (Fig. 2). As is already well known, this immunosuppressive effect is dose dependent (data not shown) and exhibits a magnitude of 80% at 1 μM for Tat and at 0.03 μM for gp160. HIV-1 $p24^{gag}$ (p24) (0.42 μM), Nef (1 μM), and Vpr (1 μM) proteins were used as controls since they were devoid of any immunosuppressive activity even at high concentrations. More interestingly, cells activated and cultured in the presence of glutaraldehyde-inactivated Tat, heat-inactivated Tat, or gp160 showed normal proliferation, as was the case for the control cells without any added HIV-1 proteins. This result supports the assumption that the immunosuppressive activity of the HIV-1 proteins Tat and gp160 is related to their biologically active forms and is not only dependent on the amino acid sequence, since the amino acid sequence is not modified by the inactivation process. The inactivated proteins remained antigenic and immunogenic as ascertained by enzyme-linked immu-

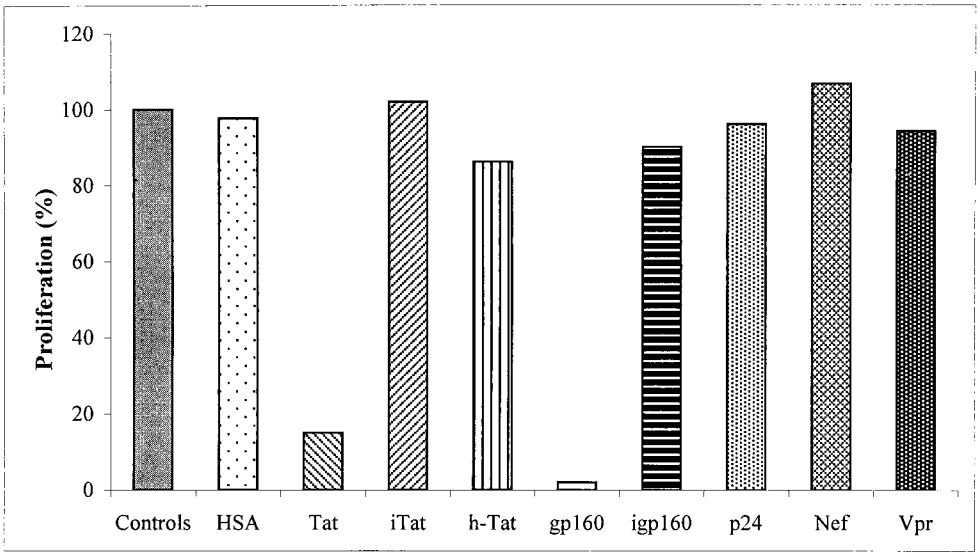

FIG. 2. Inhibition of T-cell proliferation by HIV-1 Tat and gp160. SEB-activated PBMCs were cultured for 3 days in the presence of HIV-1 gp160 (0.03 μM) or Tat protein (1 μM), or p24 (0.42 μM) or Nef (1 μM), or Vpr (1 μM) as HIV-1 protein controls, or HSA (0.03 μM). Concomitantly, cell proliferation was measured in the presence of iTat, h-Tat, or igp60 to corroborate that the anti-proliferative effects of the tested HIV-1 proteins are associated with the native forms of Tat and gp160 and not only to their amino acid sequences, since the inactivation procedures used preserve the protein structures (as measured by ELISA with specific antibodies) even if they inactivate the biological activities of the proteins.

nosorbent assay (ELISA) with the native antigens for antibody detection (Fig. 2).

HIV-1 gp160- and Tat-induced oxidative stress and immunosuppression. To investigate ROS production (in our case, mostly H_2O_2 production) and its compartmentalization, large number of cells usually have to be processed to obtain cell extracts by sonication and centrifugation. These treatments must be carried out for a relatively long period of time (about 30 min) compared to the half-life of H_2O_2 (which has the longest half-life compared with that of $O_2^{\cdot -}$ or $OH^{\cdot}$), which may impair the interpretation of results. Consequently, we have developed a method by using ultramicroelectrodes (electrodes of micrometer dimensions) that permitted us to detect superoxide derivatives such as H_2O_2 by a specific electrochemical device after introduction of a microcapillary (<0.5 μm) into a discrete site of a single living cell (Fig. 1). Figure 3 shows that the cellular response exhibits a peak shape with a maximum height (in picoamperes) reached in less than 1 s and a total area (electric charge, Q, in picocoulombs) spanned in less than 40 s. The total amount of the H_2O_2 detected is in the range of femtomoles, i.e., about 10 mM versus cellular volume. This "concentration," calculated for various human fibroblast cell lines (normal, simian virus 40 transformed, and *Xeroderma pigmentosum*) as well as for human PBMCs, isolated lymphocytes, and promonocytic cell lines, cannot preexist within a living cell because its magnitude is probably incompatible with life. Consequently, this H_2O_2 production should be considered an active neoproduction due to the membrane stress at the time of cell puncture. This has led us to consider microcapillary introduction as a model that mimics effects similar to those due to particle introduction (virus, bacteria, asbestos) into a cell and to investigate the possible role of the cytoplasmic membrane NADPH oxidase in these effects. Incubation of cells in the presence of various inhibitors of the activation of different components of NADPH oxidase prompted us to postulate that an NADPH oxidase-like enzyme may at least in part be involved in the observed H_2O_2 generation.

In the present study we used lymphocytes from healthy individuals to evaluate the significance for HIV-1-associated immunopathogenesis of pro-oxidant conditions and the possible NADPH oxidase dependent-inflammatory response which are induced by the immunosuppressive isolated HIV-1 proteins Tat and gp160.

Figure 3 shows the mean values for peak current and peak charge (surface area), as detected by our ultramicroelectrode assay, for various experiments with the HIV-1 proteins p24 (0.42 μM), Tat (1 μM), gp160 (0.03 μM), Nef (1 μM), and Vpr (1 μM) and human serum albumin (HSA) (0.03 μM). It can be concluded from this that activated lymphocytes from healthy individuals which have been incubated with HSA, p24, Nef, or Vpr exhibit a peak current and a peak charge which are similar to those of the control activated cells. In contrast, cells incubated with Tat or gp160 exhibit a peak current and a peak charge which are about two times higher than those of control cells. The redox state disorders as well as immunosuppressive effects exhibited by either Tat or gp160 are associated with their native form since igp160, h-Tat, and iTat do not induce H_2O_2 production increase (Fig. 3b and c) or suppressive effects (Fig. 2), as was the case with the native proteins. The reported data are in agreement with the observation that HIV-1 Tat and gp160 proteins contribute to the hyperactivation of the cellular inflammation state. Furthermore, responses to Tat and gp160 are dose dependent: at concentrations of 5 μg/ml of Tat or 2.5 μg/ml of gp160, H_2O_2 production was not affected.

Effects of PAO on HIV-1 Tat- and HIV-1 gp160-induced oxidative stress. In order to determine whether NADPH oxidase or NADPH oxidase-like enzymes were possible targets for the amplified oxidative stress observed either before or after incubation of cells with Tat or gp160 and induced by microcapillary introduction, we have studied the effect of PAO, a known specific NADPH oxidase inhibitor (22). We have first verified that at a concentration of 25 μM PAO inhibits 75 to 85% of the oxidative stress response of circulating activated leukocytes. As we have previously shown with HIV-1-infected macrophages, PBMCs, and NF-κB induction in U1 activated cells (3), 25 μM PAO decreased the level of Tat- or gp160-induced H_2O_2 production to that of activated control cells (Fig. 4). These results show that HIV-1 immunosuppressive

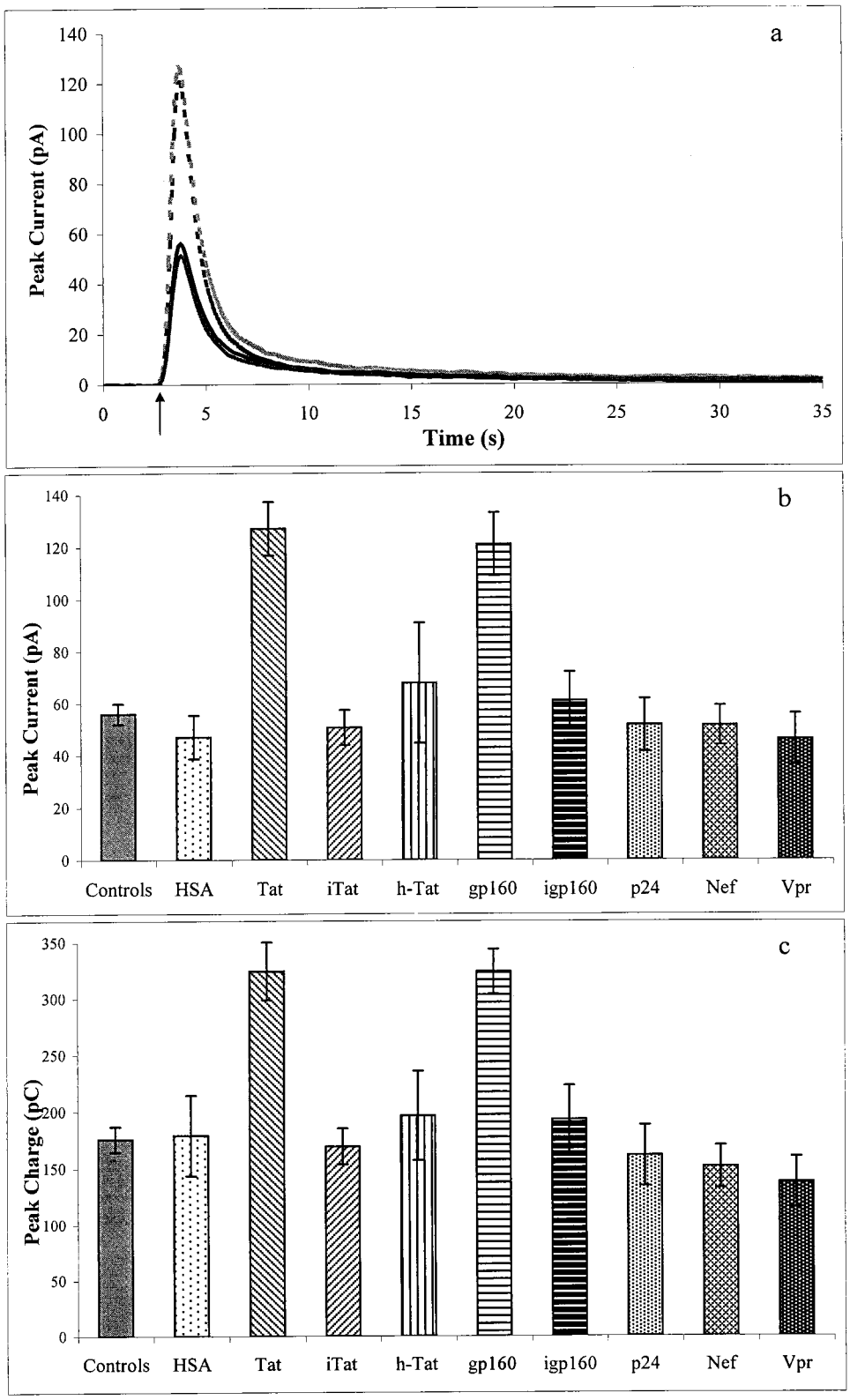

FIG. 3. Electrochemical detection of H_2O_2 production by immobilized activated human T cells incubated (15 h) with HIV-1 p24 (0.42 μM), Nef (1 μM), Vpr (1 μM), gp160 (0.03 μM), Tat (1 μM), or HSA (0.03 μM). (a) Kinetics of peaks obtained from averaged cellular responses of activated control T cells (——) and T cells incubated with p24 (——), gp160 (– – –), or Tat (– – –). (b) Effects of p24, gp160, Tat, Nef, Vpr, igp160, iTat, h-Tat, and HSA on the maximum peak current detected by amperometry on activated T cells. (c) Effects of p24, gp160, igp160, Tat, Nef, Vpr, iTat, h-Tat, and HSA on the total peak charge detected by amperometry on activated T cells. Values are means ± standard errors, and 50 to 80 cells were tested for each sample.

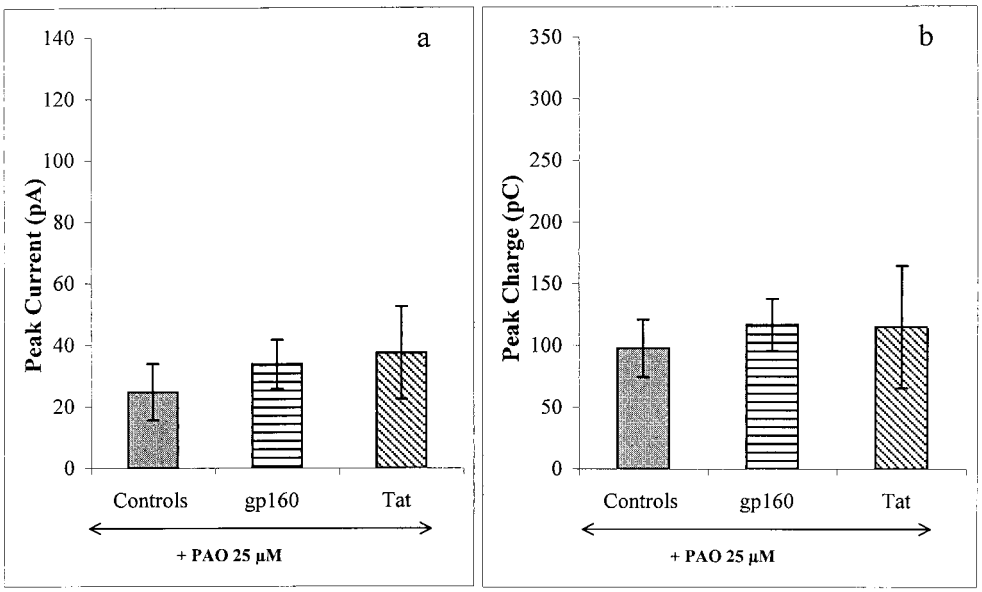

FIG. 4. Effects of PAO (1-h incubation, 25 μM concentration) on the maximum peak current (a) and the total peak charge (b) detected by amperometry on activated normal T cells and on T cells incubated with gp160 or Tat. Values are means ± standard errors.

Tat and gp160 proteins may influence cellular metabolism, at least in part, through the membrane NADPH oxidase of lymphocytes, which enhances cellular oxidative response.

DISCUSSION

The increase of oxidative stress in activated PBMCs as in PBMCs originating from HIV-1 patients is consistent with the involvement of oxidative stress in the cellular immune response (3, 20). Furthermore, it has been well documented by several groups of investigators that virus production is related through NF-κB activation to ROS level (1, 3, 13, 17).

Herewith, we show that HIV-1 Tat and gp160 are associated with a very early increase of H_2O_2 production as measured by our ultramicroelectrochemical technique. As expected, heat-denatured Tat as well as nonimmunosuppressive HIV-1 proteins, such as Nef, Vpr, and $p24^{gag}$, and the unrelated HSA did not increase the oxidative stress response (Fig. 3). Our studies were undertaken on the basis of the regulatory action of Tat protein in HIV-1 production by activation of NF-κB and on the involvement of Tat and gp160 in apoptosis as observed in the course of HIV infection for both infected and uninfected cells (28).

In this connection, it should be stressed that Tat can, at low doses, increase the production of proinflammatory chemokines MIP1α, MIP1β, and Rantes, whereas at high doses, it increases that of the immunosuppressive cytokine alpha interferon (38, 39). These elements are constitutive of the activation state of the immune cell, which is in part characterized by production of $O_2^{\cdot -}$ and/or H_2O_2, and thus mimic the condition of the inflammatory cell (3, 4, 32).

Since the inactivated Tat and gp160 molecules did not induce immunosuppression or enhance oxidative stress, they can be used as immunogens to induce specific antibodies and counteract the damaging effect of infection (16). We have previously shown that Tat inhibited cell proliferation (immunosuppression) by 85%, which inhibition could be counteracted by treating Tat-treated cells with anti-Tat. In the present study we determined that the Tat-induced increase of oxidative stress can also be abolished by Tat treatment with anti-Tat antibodies (data not shown). This latter result is consistent with the assumption that the Tat-induced immunosuppression (inhibition of cell proliferation) is related to the capacity of Tat to increase oxidative stress.

Herewith, it has also been shown that the redox imbalance is correlated in part to an increase of NADPH oxidase activity, since PAO, a well-known inhibitor of this enzyme (19), could restore the cell redox state that was disturbed by Tat or gp160 action. We are currently investigating whether the cellular redox state impairment by HIV-1 Tat and gp160 can be reverted by the use of NADPH oxidase inhibitors at physiological concentrations.

Finally, these results suggest that (i) the mechanisms leading to HIV-1-associated immunosuppression should be related, at least in part, to the overall abnormal cellular oxidative stress, and (ii) new ways of combatting immunosuppression may involve the use of drugs quenching the oxidative stress induced by the effect of gp160 and Tat proteins in the course of HIV-1 infection.

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