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Palmitic acid induces IP-10 expression in human macrophages via NF-κB activation

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Abstract

It is now recognized that cross-talk between adipocytes and adipose tissue stromal cells such as macrophages contributes to local and systemic inflammation. One factor from adipocytes that may participate in this interaction and that is frequently elevated in inflammatory conditions such as obesity, insulin resistance, and type 2 diabetes is free fatty acids (FFA). To investigate the potential for FFA to enhance macrophage inflammation, we exposed U937 macrophages to physiological levels (150 μM) of FFA. Palmitic acid (PA), the predominant saturated FFA released from adipose tissue, but not unsaturated FFA, induced an ~6-fold ($p < 0.05$) increase in IP-10 gene expression (and 2- to 4-fold increases in IL-8, MCP-1, COX-2, and MIG). PA also induced an ~2-fold increase ($p < 0.05$) in active NF-κB, and two structurally distinct NF-κB inhibitors effectively blocked PA-induced IP-10 gene expression. Conditioned medium from PA-treated cells increased lymphocyte migration 41% ($p < 0.05$) which was significantly reduced by IP-10-neutralizing antibody. These results suggest that elevated concentrations of PA commonly present in obese and insulin resistant individuals can increase NF-κB-mediated expression of IP-10 in macrophages. These events in turn may lead to an increasing feed-forward loop of chronic inflammation.

Keywords: Macrophages; Free fatty acids; Inflammation; Obesity; NF-κB; IP-10; Palmitic acid

Obesity is characterized by adipose tissue inflammation, and many of the pro-inflammatory factors secreted from adipose tissue derive from stromal cells such as macrophages [1]. In addition, monocytes/macrophages newly recruited to adipose tissue appear to demonstrate greater production of pro-inflammatory factors and decreased expression of protective factors than more quiescent resident macrophages [2]. The importance of macrophages to adipose tissue inflammation, as well as to local and systemic insulin resistance, has been demonstrated in several recent animal studies [1,3,4].

This pro-inflammatory interaction between macrophages and adipocytes in adipose tissue may also be relevant for vascular disease and may help explain the increased CVD risk with obesity. Adipose tissue inflammation appears to contribute to systemic inflammation, and specifically to enhanced monocyte activation. Recent studies also suggest that perivascular fat inflammation is associated with, and perhaps contributes to, vascular disease [5]. Therefore, it is becoming clear that both adipocytes and macrophages may contribute to the chronic inflammatory processes in adipose tissue [1,4,6,7] and that this interaction may exacerbate a variety of chronic inflammatory conditions.

We recently reported that treatment of 3T3-L1 adipocytes with conditioned medium from RAW264.7 macrophages resulted in the upregulation of several pro-inflammatory factors including free fatty acids (FFA) [8]. The present study examined the hypothesis that FFA may in turn feed back to modulate the inflammatory state of *human* macrophages. While saturated FFAs have been

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reported to have unique pro-inflammatory effects compared to unsaturated FFAs in several murine models, possibly via the endotoxin receptor, TLR4 [9], these effects have not been carefully studied in human cells. In fact, some studies have noted significant differences in TLR signaling between murine and human macrophages [10]. Our own preliminary data suggested that one such difference is that human, but not murine, macrophages substantially upregulate IP-10 expression in response to TLR4-mediated stimuli such as LPS.

There is growing recognition that the chemokine IP-10 plays important roles in chronic inflammatory conditions such as atherosclerosis [11], but the potential roles for FFA-induced IP-10 in these conditions have not been previously examined. We therefore examined the effects of physiological levels of saturated fatty acids on production of IP-10 and other pro-inflammatory factors by human macrophages.

Materials and methods

Reagents. The proteasome inhibitor MG132 (1 μM) was from Biomol (Plymouth Meeting, PA), and Pyrolidinedithiocarbamate (PDTC; 25 μM) was from Sigma (St. Louis, MO). Human TNF-α and a monoclonal anti-human blocking antibody to IP-10 was purchased from R&D Systems (Minneapolis, MN). Recombinant human IP-10 protein was obtained from RDI (Flanders, NJ). All reagents not otherwise specified were from Sigma (St. Louis, MO).

Cells and cell culture. Human U937 monocytes were obtained from the American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 (Invitrogen, Gaithersburg, MD) supplemented as recommended by the vendor. Monocytes were differentiated into macrophages using 12 ng/mL phorbol ester overnight. Lymphocytes were isolated from the blood of healthy volunteers by gradient centrifugation [12] and purified by overnight growth in RPMI containing 10% human serum.

Free fatty acid preparation. Sodium salts of palmitic acid (PA), dodecanoic (lauric) acid, oleic acid, linoleic acid, eicosapentaenoic acid, and arachidonic acid were from lots demonstrated to be >99% pure by gas chromatography and were tested for endotoxin contamination using the Pyrogent assay kit (Cambrex, Gaithersburg, MD); only lots containing <5 pg endotoxin per mole FFA were used on cells. FFAs were dissolved to 40 mM in 0.1 N NaOH 70% ethanol at 70 °C. We have found that this solvent neutralizes >10 ng/mL endotoxin (unpublished data). Fatty acids were purged with nitrogen gas and stored in small aliquots at −80 °C. Aliquots were used within four weeks and freeze-thawed no more than twice.

Exposure of cells to free fatty acids. FFA preparations were thawed and briefly warmed immediately before addition to cell culture media. FFA were added to final concentrations of 50, 100, and 150 mM in RPMI 1640 media (containing 10% FBS, 0.3 mg/mL L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) and allowed to complex with the albumin present in the FBS at 37 °C for 1 h. The final FFA/albumin molar ratio was approximately 2:1. For individual experiments, U937 macrophages were treated in media containing 0–150 μM FFA, and were subsequently maintained under standard cell culture conditions for 4–72 h.

Generation of conditioned media and cytokine protein quantification. Macrophages were maintained under control conditions or treated with 150 μM PA for 48 h, washed and subsequently incubated in fresh, PA-free media for 6–48 h. Conditioned media were collected from these cultures at the appropriate time points, then aliquoted and snap-frozen to −80 °C. Aliquots were thawed only once prior to analysis or use on cultured cells. IP-10 protein concentrations in conditioned cell culture media were analyzed by ELISA (R&D Systems) following manufacturer's instructions. Samples outside the range of the standard curve were diluted in ELISA sample buffer and re-analyzed.

Measurement of NF-κB activation. Following treatment of cells with PA for the indicated time period, cells were fractionated to extract nuclear and cytoplasmic proteins using the hypotonic lysis method of Berg et al. [13]. Equal amounts of protein from each lysate were analyzed for NF-κB activation using a TransAM™ NF-κB p65 assay kit (Active Motif, Carlsbad, CA) following manufacturer's instructions. Phosphorylation of IκBα in the cytoplasmic fraction was analyzed by Western blot, using standard laboratory methods and a specific monoclonal antibody against phosphorylated IκBα (clone B9; Santa Cruz Biotechnology, Santa Cruz, CA).

Lymphocyte migration assay. Primary human lymphocyte migration was measured using Micro Chemotaxis Transwell plates (polycarbonate membrane, pore size 5.0 μm; Costar, Corning, NY). PA-free conditioned media from macrophages were prepared as described above in *Generation of conditioned media and cytokine protein quantification*. Cell migration was stimulated under standard cell culture conditions with various conditioned media (described below in Results), and cells that had migrated through the membrane after 90 min were counted using a Coulter counter.

Reverse transcription-PCR and real-time quantitative PCR analysis. Total RNA was isolated, converted to cDNA, and analyzed by real-time quantitative PCR using SYBR Green chemistry (qPCR) with reagents and equipment from Bio-Rad (Hercules, CA). Primer pairs were: IP-10 sense, AGGAACCTCCAGTCTCAGCA; IP-10 antisense, CAAAATTGGC TTGCAGGAAT and 18 s sense, GGACTTCGAGCAAGAGATGG; 18 s antisense, AGCACTGTGTTGGCGTACAG. Quantification of gene expression was performed by the δδCt method [14].

Statistics and data analysis. Data are expressed as means ± SD. Data were analyzed by ANOVA using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Experiments demonstrating a difference between groups by ANOVA were subsequently analyzed by post hoc *Z*-statistics (corrected for multiple comparisons) when experiments compared samples that had been normalized to controls (e.g., real-time PCR), or by Bonferroni's *t*-tests when experiments compared different means (e.g., migration assays). All experiments were repeated a minimum of three times. Results were considered significant at $p < 0.05$.

Results

Expression of pro-inflammatory cytokine mRNA following treatment of macrophages with FFAs

We first exposed U937 macrophages to the major individual FFAs found in human plasma (Fig. 1) at a concentration of 150 μM (except arachidonic acid, which was toxic to macrophages above 50 μM). The saturated FFAs palmitic and lauric acid induced a 6-fold increase in IP-10 mRNA after 48 h. In contrast, the unsaturated FFAs either reduced IP-10 mRNA levels or had no significant effect. We further investigated the effects of palmitic acid (PA) because it is the most abundant saturated FFA in human tissue and plasma.

PA treatment of macrophages induces time- and concentration-dependent increases in IP-10 mRNA

Macrophages exposed to 150 μM PA for time periods of 6–72 h demonstrated increased IP-10 expression in a time-dependent manner, reaching a maximum at 48 h, but remaining elevated for at least 72 h (Fig. 2A). Expression of several other pro-inflammatory genes was also examined

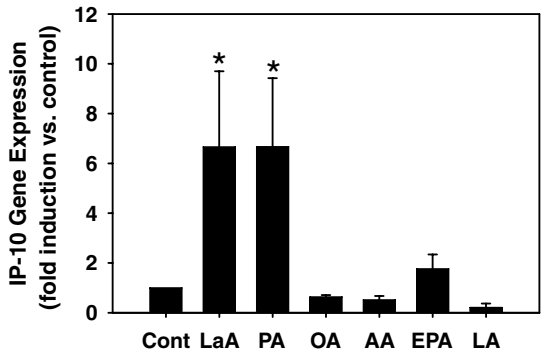

Fig. 1. Saturated but not unsaturated FFAs induce expression of IP-10. U937 macrophages were treated with 150 μM FFA for 48 h. The saturated FFAs palmitic (PA) and lauric (LaA) acid induced IP-10 gene expression, but the unsaturated FFAs oleic (OA), eicosapentaenoic (EPA), linoleic (LA), and arachidonic acids (AA; 50 μM, limited by toxicity) did not. Data are IP-10 gene expression normalized to vehicle controls; $n = 3$ experiments; $^{*}p < 0.05$ vs. untreated controls.

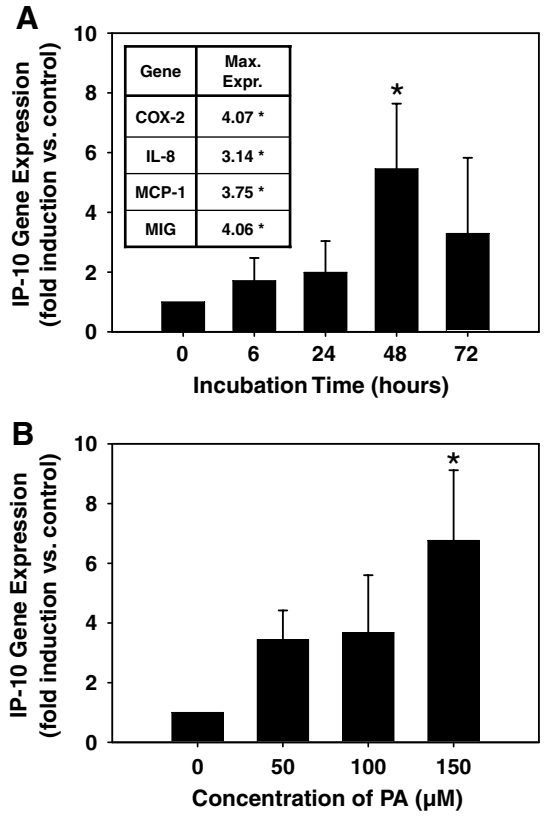

Fig. 2. Exposure of macrophages to PA induces time- and dose-dependent increases in pro-inflammatory cytokine gene expression. (A) U937 macrophages were treated with 150 μM PA for 0 (control) to 72 h. Exposure to PA for 48 h resulted in a maximum 6-fold increase in IP-10 mRNA expression. Inset: Exposure of macrophages to PA resulted in 3- to 4-fold induction of other pro-inflammatory cytokines (times and PA concentrations eliciting maximum expression not shown). Data are fold induction vs. same-time-point control; $n \geqslant 3$ experiments; $^{*}p < 0.05$ vs. untreated controls. (B) Macrophages were exposed to 0–150 μM PA for 48 h. Exposure of cells for 48 h resulted in a dose-dependent increase in expression of IP-10 with a maximum at 150 μM. Data are fold induction vs. control; $n = 3$ experiments; $^{*}p < 0.05$ vs. untreated controls.

and modest increases were seen for COX-2, MCP-1, IL-8, and MIG (Fig. 2A, inset). Macrophages exposed to a range (0–150 μM) of PA concentrations for 48 h demonstrated a dose-dependent increase in IP-10 expression, reaching a maximum at 150 μM PA (Fig. 2B). PA concentrations much above 150 μM for 48 h initiated mild toxicity. Subsequent experiments, unless specifically stated, were therefore conducted at 150 μM concentration.

IP-10 gene expression is accompanied by protein secretion

We next investigated whether IP-10 mRNA expression was accompanied by protein secretion. Because a significant rise in IP-10 mRNA did not occur until after 48 h of

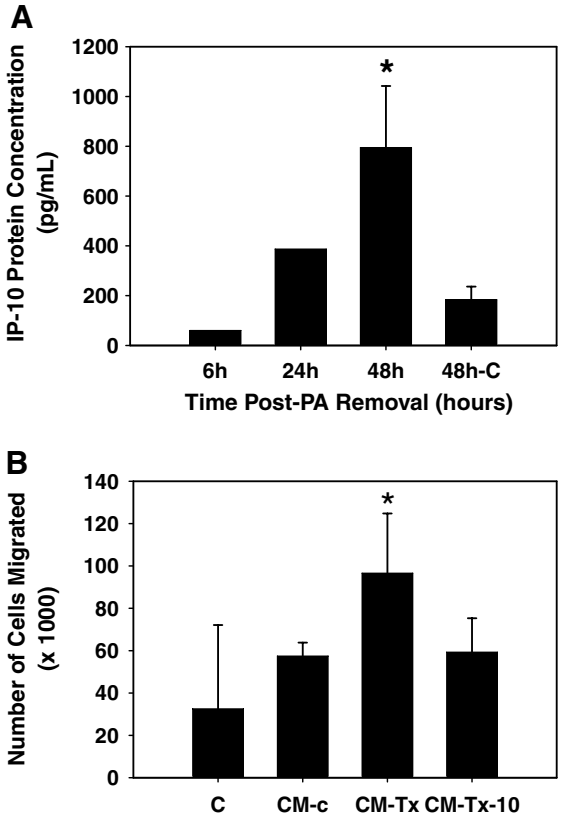

Fig. 3. Exposure of macrophages to 150 μM PA for 48 h. Resulted in increased secretion of bioactive IP-10 protein. (A) U937 macrophages were treated with PA for 48 h then subsequently given fresh media. These conditioned media (CM) were collected at 6, 24, and 48 h after PA treatment; controls were untreated cells given fresh media for 48 h (48-C). IP-10 protein continued to accumulate in CM from PA-treated macrophages for up to 48 h after removal of PA. Data are IP-10 protein measured by ELISA (pg/mL); $n = 3$ experiments; $^{*}p < 0.05$ vs. 48 h controls. (B) Freshly isolated human lymphocytes were placed in Transwell® permeable supports and incubated with PA-free CM from U937 macrophages for 90 min at 37 °C. Controls (C) were RPMI/0.1% BSA; CM-c = conditioned media from unstimulated macrophages; CM-Tx = conditioned media from macrophages previously exposed to PA for 48 h; CM-Tx-10 = CM-Tx pre-treated with blocking monoclonal antibodies to IP-10. Data are number of cells that migrated through the Transwell membrane; $n = 4$ experiments; $^{*}p < 0.05$ vs. vehicle control.

PA treatment (Fig. 2B), we chose to study IP-10 protein secretion after this time point. We therefore treated macrophages with PA for 48 h, washed the cells in PBS to remove residual PA, and provided them with fresh media; controls were treated identically. Cells were incubated in this PA-free media for 6–48 h, and media were collected for analysis by ELISA and use in functional assays. This procedure had the added benefit of producing conditioned medium enriched in IP-10 for use in subsequent functional assays (described below) without the confounding influence of a direct PA effect. In macrophages that had been exposed to PA, there was a steady increase in IP-10 protein levels with time (Fig. 3A).

PA-induced IP-10 protein is biologically active

Although IP-10 protein has several functions, it is best known as a potent chemoattractant for T-lymphocytes [15]. We therefore tested whether IP-10 protein in the conditioned medium following treatment of macrophages for 48 h with PA could increase lymphocyte migration. As shown in Fig. 3B, this conditioned medium induced a significant increase ($p < 0.05$) in lymphocyte migration compared to medium from untreated cells. This migration was effectively inhibited by the co-addition of an IP-10 neutralizing antibody (Clone 33036, R&D Systems), but not by nonspecific IgG_1 (data not shown), providing evidence that IP-10 was a major factor in the observed enhancement of lymphocyte migration.

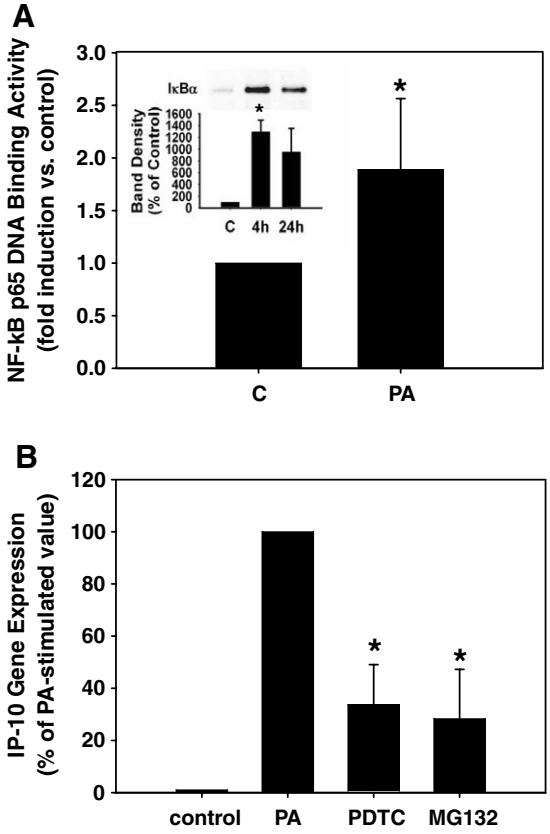

Fig. 4. The NF-κB pathway is involved in the response of macrophages to PA. (A) U937 macrophages were treated with 0 (C) or 150 μM PA and active NF-κB in cell nuclei was measured by immunoassay. Exposure of macrophages to 150 μM PA for 4 h resulted in increased translocation of active NF-κB p65 to the nucleus. Data are fold induction of p65 translocation, normalized to untreated controls; $n = 3$ experiments; $^{*}p < 0.05$ vs. untreated controls. Inset: Phosphorylation of the endogenous NF-κB inhibitor IκBα. Data are band density of phosphorylated IκBα protein, normalized to untreated controls; $n = 4$ experiments; $^{*}p < 0.05$ vs. untreated controls. (B) Inhibitors of NF-κB activation (PDTC and MG132) significantly blocked PA-induced IP-10 gene expression in U937 macrophages. Data are percent of PA-induced IP-10 gene expression; $n = 3$ experiments; $^{*}p < 0.05$ vs. PA-treated cells.

Exposure of macrophages to PA activates NF-κB

Following exposure of macrophages to 150 μM PA for 4 h, NF-κB activity was elevated nearly 2-fold over unstimulated controls (Fig. 4A). A Western blot of cytoplasmic lysates from the same experiments (Fig. 4A, inset) using phosphospecific antibodies demonstrated that this was accompanied by phosphorlyation (and presumed ubiquitination and proteasomal degradation) of IκBα. Phosphorylation was strongest after 4 h of PA treatment, and continued through at least 24 h. Two structurally and functionally distinct pharmacological NF-κB inhibitors were used to demonstrate the involvement of NF-κB activity in PA-induced IP-10 expression (Fig. 4B). Both PDTC and MG132 significantly inhibited IP-10 mRNA expression in response to PA, strongly suggesting that NF-κB is required for saturated FFA-mediated inflammation and IP-10 expression by macrophages.

Discussion

IP-10 is a member of the CXC chemokine family, which includes other factors such as MIG, I-TAC, IL-8, and MIP-2, and is produced by a variety of cell types, including inflammatory cells such as monocytes, macrophages, and lymphocytes [11,16]. Human serum levels of IP-10 range from 20–400 pg/ml, with the higher values found most commonly among individuals with chronic inflammatory conditions such as rheumatoid arthritis, HIV infections, and atherosclerosis [11,17–20]. Recent studies have also described increased plasma levels of IP-10 in the early and subclinical stages of Types 1 and 2 diabetes [21–23], suggesting that this protein may be a causal factor in the development of these conditions, or at least a consequence of their underlying pathology. Although generally recognized as an important chemoattractant for lymphocytes [15], and monocytes [16], IP-10 also enhances adherence of inflammatory cells to endothelial cells [16,17] and suppresses angiogenesis [16,24]. There is thus a growing appreciation for the potential role of IP-10 in a variety of chronic inflammatory conditions, including diabetes and atherosclerosis [20,22,23].

The present study demonstrated that exposure of human macrophages to saturated FFA, but not unsaturated FFA,

induces IP-10 gene expression in a time- and dose-dependent manner. Maximum expression of IP-10 occurred at around 150 μM of PA, a high physiologic plasma concentration that would commonly be present in individuals with obesity and insulin resistance. Although lauric acid at these concentrations was equally capable of stimulating IP-10 expression, such concentrations of lauric acid would not be physiological.

We chose lymphocyte migration, a classic function of IP-10, to demonstrate that the IP-10 protein secreted in response to PA was indeed bioactive. Although the conditioned media from the PA-treated macrophages undoubtedly contained several bioactive factors, and our own data demonstrated the induction of several other inflammatory genes, specific blocking antibodies to IP-10 could inhibit a substantial portion of the lymphocyte migration, consistent with a role for SFA-induced increases in IP-10 secretion in tissue inflammation.

Our results also demonstrated that palmitic acid-induced activation of NF-κB, a transcription factor known to regulate expression of IP-10 [25], was required for upregulated IP-10 expression. As NF-κB is a "master regulator of inflammation" [25], it is quite likely that the inflammatory effects of saturated fatty acids on human macrophages go far beyond upregulation of IP-10. In support of this notion, PA induced several other inflammatory products (Fig. 2A, inset). These results are consistent with several recent studies that have suggested fatty acids may activate NF-κB through activation of TLR4 [6,9]. Of relevance, in murine macrophages and in human HEK 293 cells transfected with TLR4, saturated, but not unsaturated, fatty acids increased COX-2 and nitric oxide production in a TLR4-dependent fashion [9]. IP-10 is also a well-recognized product of MyD88-independent TLR4 activation, and was increased by the classical TLR4 ligand, bacterial endotoxin, in our own studies of U937 macrophages (data not shown). However, fatty acids have been shown to stimulate other pro-inflammatory pathways, including generation of reactive oxygen species [26], and activation of MAPK pathways [27], and although consistent with activation of TLR4, our results do not exclude these other inflammatory pathways.

In summary, the present study described increased production of IP-10 by human macrophages exposed to relevant physiological concentrations of saturated fatty acids, and demonstrated that this activation required NF-κB activity. The concentrations of PA required to induce this response were comparable to levels that would commonly be present in insulin resistant individuals. Our laboratory and others [6,8] have previously demonstrated that murine macrophages can induce adipocyte inflammation *in vitro*. The results of the present study suggest one possible mechanism whereby adipocytes can also mediate inflammation (and NF-κB activation) of human macrophages, through their production of free fatty acids. Saturated FFA-induced production of IP-10 by human macrophages, a novel pro-inflammatory response not seen in murine macrophages, could lead to an increasing feed-forward loop and a potential explanation for the chronic inflammation associated with obesity-related human pathologies, such as type 2 diabetes and CVD.

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