Inhibition of vagally mediated immune-to-brain signaling by vanadyl sulfate speeds recovery from sickness

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To the ill patient with diabetes, the behavioral symptoms of sickness such as fatigue and apathy are debilitating and can prevent recuperation. Here we report that peripherally administered insulin-like growth factor 1 (IGF-1) attenuates LPS-dependent depression of social exploration (sickness) in nondiabetic (*db*/+) but not in diabetic (*db*/*db*) mice. We show that the insulin/IGF-1 mimetic vanadyl sulfate (VS) is effective at augmenting recovery from sickness in both *db*/+ and *db*/*db* mice. Specifically, peak illness was reached at 2 h for both VS and control animals injected with LPS, and VS mice recovered 50% faster than non-VS-treated animals. Examination of the mechanism of VS action in *db*/+ mice showed that VS paradoxically augmented peritoneal macrophage responsivity to LPS, increasing both peritoneal and *ex vivo* macrophage production of IL-1β and IL-6 but not TNF-α. The effects of VS in promoting recovery from sickness were not restricted to LPS, because they were also observed after direct administration of IL-1β. To explore the possibility that VS impairs immune-to-brain communication via vagal afferents, the vagally mediated satiety-inducing effects of cholecystokinin 8 were tested in *db*/+ mice. Cholecystokinin decreased food intake in saline-injected mice but not in VS-treated mice. VS also inhibited LPS-dependent up-regulation of IL-1β and IL-6 mRNA in the brain, while increasing by 50% the cerebral expression of transcripts of the specific antagonist of IL-1 receptors IL-1RA and IL-1R2. Taken together, these data indicate that VS improves recovery from LPS-induced sickness by blocking vagally mediated immune-to-brain signaling and by up-regulating brain expression of IL-1β antagonists.

neuroimmunity | sickness behavior | type 2 diabetes | vanadium

Stimulation of the peripheral innate immune system by the cytokine inducer LPS causes expression of proinflammatory cytokines in the brain, and this response is associated with development of sickness behavior (1). We have recently shown that type 2 diabetic (*db*/*db*) mice display an enhanced and more prolonged episode of sickness in response to LPS and IL-1β (2). How diabetes alters brain–immune interactions however, is still unclear. Insulin resistance is a critical component of type 2 diabetes and is likely responsible for initiation (3). Strategies to maximize insulin sensitivity, increase insulin action, and/or inhibit insulin counterregulation might be expected to neutralize the deleterious effect of type 2 diabetes on superimposed illness. One agent with antidiabetic properties is vanadium (V). V is a naturally occurring element found in soil and rocks at a concentration of ≈150 ppm (4). It does not normally exist in elemental form but is bound most commonly to oxygen, sodium, chloride, and sulfur (5). V has six oxidation states (1− through 5+), with V + 4 and V + 5 being most common in the body (6). The average human intake of V is 10–20 μg/day, mostly from plant material (5) in the form of sodium metavanadate, sodium orthovanadate, V pentoxide, and vanadyl sulfate (VS) (7). Although its specific function in humans is unclear, dietary V is required for proper growth and development (8).

Some common over-the-counter vitamin supplements contain V (9), and V is claimed to mitigate hyperglycemia (10, 11) and build muscle mass (12, 13). These insulin and IGF-1 mimetic properties of V may be linked to its ability to inhibit protein tyrosine phosphatases (14) that normally provide negative feedback to protein kinases within the insulin/IGF-1 signaling cascade (2, 15–22). V compounds adopt a trigonal bipyramidal structure that mimics the transition state of the phosphoryl transfer reaction, thereby acting either as a competitive inhibitor (VS) or an oxidizer (peroxovanadate complexes) of the conserved cysteine residue in the protein tyrosine phosphatase loop (23). V derivatives can directly activate members of the mitogen-activated protein kinases (24–26) and induce phosphatidylinositol 3′-kinase association to activating docking molecules like insulin receptor substrates (IRSs) (27). In combination with insulin, V compounds can augment/prolong insulin receptor and IRS-1 tyrosine phosphorylation (28) and enhance insulin-induced glucose transport (29). Furthermore, VS may have antiinflammatory actions in diabetes, because it decreases nitric oxide production in peritoneal macrophages derived from streptozotocin-treated mice (30).

Systemic activation of the innate immune system characterized by chronic low-grade inflammation appears necessary to the pathogenesis of type 2 diabetes and many of its complications (31–35). Importantly, the innate immune system communicates immune status to the CNS to induce sickness, which refers to the coordinated set of behavioral modifications that occur during infection (36). These evolutionarily conserved adaptive behaviors reflect a reorganization of motivational priorities that maximize immune efficiency in fighting infection (37). Proinflammatory cytokines IL-1β, IL-6, and TNF-α (38–42), produced by antigen-presenting cells in reaction to innate immune challenge, are principally responsible for sickness. Mechanistically, peripherally administered LPS induces synthesis and expression of IL-1β, IL-6, and TNF-α in brain (38, 39). Brain production of IL-1β in response to peripheral inflammatory stimuli likely originates in the choroid plexus and circumventricular organs (43), but brain microglial cells and perivascular/meningeal macrophages (44) can also produce IL-1β. Brain-produced IL-1β is essential for sickness as shown by IL-1 inhibition (45) and knockout (46, 47) studies where its action on brain structures such as basolateral amygdala, hypothalamus, and area postrema (38, 48) elicits sickness. We have shown that anti-inflammatory agents like IGF-1 reduce sickness induced by LPS (42, 49). Here we show that VS facilitates recovery from cytokine-induced sickness by modulating neural transmission of the peripheral immune message to the brain.

Abbreviations: IGF-1, insulin-like growth factor-1; IL-1RA, IL-1 receptor antagonist; i.c.v., intracerebroventricular; IL-1R2, type-2 IL-1 receptor; V, vanadium; VS, vanadyl sulfate; SE, social exploration; CCK, cholecystokinin.

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Experimental Procedures

Materials. All reagents and chemicals were purchased from Sigma, except the following: FCS (0.05 ng/ml, 0.48 units/ml endotoxin) and recombinant murine IL-1β (Atlanta Biologicals, Norcross, GA); primer pairs (Qiagen, Alameda, CA); Sybr green PCR master mix and MicroAmp optical 96-well reaction plates (PE Applied Biosystems); TRIzol (Gibco BRL Life Technologies); superscript III RNase H^- reverse transcriptase, 10 mM dNTP mix, and oligo dT primers (Invitrogen Life Technologies); RNasin Rnase inhibitor (Promega); mouse cannulas and cyanyoacrylate gel adhesive (Plastics One, Roanoke, VA); anti-mouse IL-1β (PM425B1), TNF-α (MM350C), IL-6 (MM600D), biotin-labeled anti-mouse IL-1β (MM425BB), TNF-α (MM350DB), IL-6 (MM600CB), horseradish peroxidase-conjugated streptavidin (n100), 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (n301), mouse IL-1β (SMIL1B), TNF-α (SMTNFN), and IL-6 (SMIL6) ELISA standard (Endogen, Woburn, MA); and Maxisorp-coated 96-well ELISA plates (Nalge Nunc, Rochester, NY).

Animals. All animal care and use were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, as described (50). B6.Cg-*M*+/+*Lepr*^{db} (*db*/+) and B6.Cg-+*Lepr*^{db}/+*Lepr*^{db} (*db*/*db*) mice were bred in-house from mice purchased from The Jackson Laboratory. Mice were housed in standard shoebox cages and allowed pelleted food (NIH 5K52; LabDiet, Purina Mills, Brentwood, MO) and water *ad libitum* in a temperature- (72°F) and humidity- (45–55%) controlled environment with a 12-h/12-h dark–light cycle (0700–1900 hours). Male 8- to 12-wk-old mice were used for all experiments. For VS-treated mice, VS (0.5 mg/kg) was diluted in sterile PBS to a concentration of 0.12 mg/ml and delivered i.p.

Measurement of Sickness. Sickness was quantified by using social exploration (SE), as described (2). Mice were i.p.-injected with LPS (*Escherichia coli*, O127:B8) or vehicle in a volume of 0.25 ml, as indicated. A 3- to 4-wk conspecific juvenile mouse was placed in the home cage of the adult test mouse for 10 min immediately prior to and 2, 4, 8, 12, and 24 h after treatments. Interaction between subject and juvenile was video-recorded, and time spent engaged in exploratory behavior was determined from video records. To control for mouse-to-mouse variability in baseline activity and allow comparison of relative changes in exploration levels, a preinjection (0 h) measurement was used as an internal control for each mouse. Results are expressed as percentage of baseline measurement and shown as means ± SEM.

Measurement of IL-1β, IL-6, and TNF-α. Cytokines were measured by ELISA as described (2). Absorbance was measured on an OPTImax tunable microplate reader (Molecular Devices) at 450–550 nm. The concentration of IL-1β, IL-6, and TNF-α in the samples was determined by reference to a standard curve.

Peritoneal Macrophage Isolation. As described (50), mice were killed by CO_2 asphyxiation, peritoneal lavage fluid was collected by (2×) peritoneal lavage with 5 ml of ice-cold growth media (RPMI medium 1640 supplemented with 10% FCS/1 g/liter glucose/2 g/liter sodium bicarbonate/110 mg/liter sodium pyruvate/62.1 mg/liter penicillin/100 mg/liter streptomycin/10 mM Hepes, pH 7.4). For *ex vivo* experiments, peritoneal cells were resuspended in 5 ml of hypertonic red-blood-cell lysis buffer (142 mM NaCl/1 mM $KHCO_3$/118 mM NaEDTA, pH 7.4) at room temperature for 4 min. An equal volume of cold growth media was added, followed by cell washing and resuspension in 37°C growth media. Cells were plated at 5×10^5 cells per ml. After 30 min, nonadherent cells were removed by washing twice with growth media. Remaining cells were at least 80% macrophages confirmed by CD11b staining and morphology (50).

Intracerebroventricular (i.c.v.) Administration of LPS. Mice were anesthetized with sodium ketamine hydrochloride/xylazine hydrochloride solution (80 mg/ml ketamine/12 mg/ml xylazine at 1.5 ml/kg body weight) and placed in a Kopf stereotaxic device (Kopf Instruments, Tujunga, CA). A sterile 28-gauge mouse stainless steel brain infusion cannula was implanted into the lateral ventricle. The coordinates used were 0.6 mm posterior and 1.5 mm lateral to the bregma and 2.0 mm ventral from the surface of the skull. Cannula were fixed to the skull with cyanoacrylate gel adhesive. Mice were allowed to recover for 3 days before the start of experimentation. Treatment was 100 ng of LPS (50 ng/μl) or vehicle control infused at a rate of 1 μl/min.

Cholecystokinin (CCK)-Induced Food Intake Suppression. As described (51), mice were fasted (with water available) for 16 h then i.p. administered 16 μg/kg CCK-8. Before CCK treatment, control mice received a daily i.p. injection of sterile saline (0.01 ml/g) for 2 wk to habituate them to handling and reduce stress. VS mice received i.p. saline for 1 wk, then i.p. VS for 1 wk. Food was reintroduced 5 min after CCK administration, and food intake was measured at 60 and 90 min.

RNA Extraction and Reverse Transcription. As described (2), total RNA from spleen or whole-brain samples was extracted in TRIzol reagent. All reverse transcriptase reactions were carried out in a Stratagene Robocycler Gradient 96. All RNA samples from a single experimental group were reverse transcribed simultaneously to minimize interassay variation associated with the reverse transcription reaction.

Real-Time PCR. Real-time PCR reactions were performed as described (2). PRIMER EXPRESS software (PE Applied Biosystems) was used to design appropriate primer pairs. The primer sequences used were as follows: β actin forward, GGCGCTTTTGACTCAGGATT; β actin reverse, GGGATGTTTGCTCCAACCAA; IL-1β forward, CTGTGTCTTTCCCGTGGACC; IL-1β reverse, CAGCTCATATGGGTCCGACA; TNF-α forward, ATCCGCGACGTGGAACTG; TNF-α reverse, ACCGCCTGGAGTTCTGGAA; IL-6 forward, CCAGAAACCGCTATGAAGTTCCT; IL-6 reverse, CACCAGCATCAGTCCCAAGA; IL-1β receptor antagonist forward, TTTAGCTCACCCATGGCTTCA; IL-1β receptor antagonist reverse, GCATCTTGCAGGGTCTTTTCC; type-2 IL-1 receptor forward, GCCTCATGTCTCCTACTTGCAA; and type-2 IL-1 receptor reverse, CTTTCAGGTCAGGGCACACTAGT. Real-time PCR was performed on Applied Biosystems Prism 7700 (PE Applied Biosystems) by using the Sybr Green PCR Master Mix. To normalize gene expression, a parallel amplification of endogenous and target genes was performed with Sybr green reagents. Reactions with no reverse transcription and no template were included as negative controls. Relative quantitative evaluation of target gene levels was performed by comparing ΔC_t, where C is the threshold concentration.

Statistical Analysis. Data are presented as mean ± SE. Experimental data were analyzed by two-, three-, or four-way ANOVA, depending on the experimental design with repeated measurements in the time factor where applicable. Post hoc comparisons of individual group means were carried out with the Tukey test (SAS Institute, Cary, NC). Statistical significance was denoted at $P < 0.05$.

Results

VS Improves Sickness in Response to LPS. Because IGF-1 can mitigate the sickness response to LPS (49), sickness was examined in *db*/*db* mice pretreated with IGF-1. Fig. 1*A* shows that when *db*/+ and *db*/*db* mice were challenged with or without i.p. LPS (5 μg), *db*/+ mice were significantly less sick 8 h after LPS than *db*/*db* mice (42.5 ± 6.5% reduction vs. 66.9 ± 8.9% reduction). When *db*/+ and

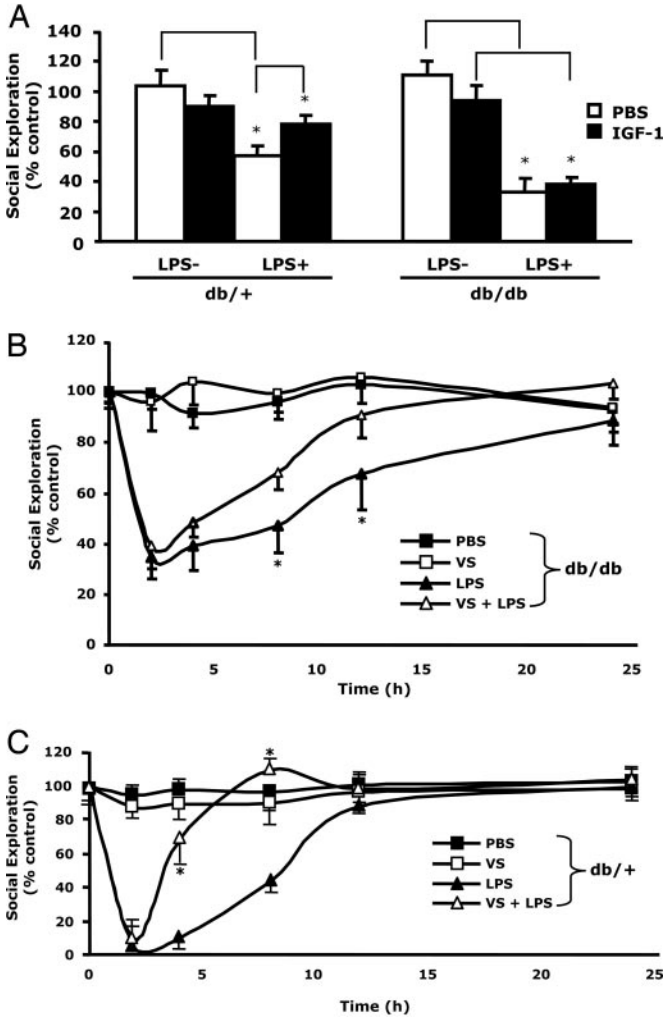

Fig. 1. VS improves sickness in response to LPS. (*A*) *db*/+ or *db*/*db* mice were pretreated with or without i.p. IGF-1 (1 μg). Sixty minutes later, LPS (5 μg) or vehicle control (PBS) was administered i.p. SE was measured at 8 h after LPS injection. Results are expressed as a percentage of the pre-LPS SE baseline measurement and shown as means ± SEM; $n = 4$ (*, $P < 0.05$). (*B*) *db*/*db* mice, as indicated, were treated with VS (0.5 mg/kg per day) or vehicle control (PBS) for 7 days before i.p. injection of LPS (5 μg). SE was measured immediately before and at 2, 4, 8, 12, and 24 h after injection. Results are expressed as percentages of the baseline measurement and shown as means ± SEM; $n = 6$. *, $P < 0.05$ VS-LPS vs. PBS-LPS. (*C*) *db*/+ were treated with VS (0.5 mg/kg) or vehicle control (PBS) for 7 days before i.p. injection of LPS (100 μg/kg). SE was measured immediately before and at 2, 4, 8, 12, and 24 h after injection. Results are expressed as percentages of the baseline measurement and shown as means ± SEM; $n = 5$. *, $P < 0.05$ VS-LPS vs. PBS-LPS.

db/*db* mice were pretreated with i.p. IGF-1 (1 μg) before LPS administration, IGF-1-treated *db*/+ mice showed little sickness at 8 h (22 ± 6.1% reduced). *db*/*db* mice were resistant (61.7 ± 4.4% reduced) to the protective effect of IGF-1 on neuroimmunity. Three-way ANOVA (phenotype × IGF-1 × LPS) revealed significant phenotype × LPS interaction [F (1, 24) = 41.86, $P < 0.0001$] and IGF-1 × LPS interaction [F (1, 24) = 24.30, $P < 0.0001$] but not a phenotype × IGF-1 × LPS interaction. No baseline difference in SE was seen in *db*/+ and *db*/*db* mice (249 ± 21 sec vs. 236 ± 16 sec). To determine whether the insulin/IGF-1 mimetic, VS, improved recovery from sickness, *db*/*db* mice were treated for 7 days with or without i.p. VS (0.5 mg/kg per day). A three-way ANOVA (VS × LPS × time) revealed significant VS × LPS × time interaction [F (5, 80) = 9.00, $P < 0.0001$] (Fig. 1*B*). VS-treated

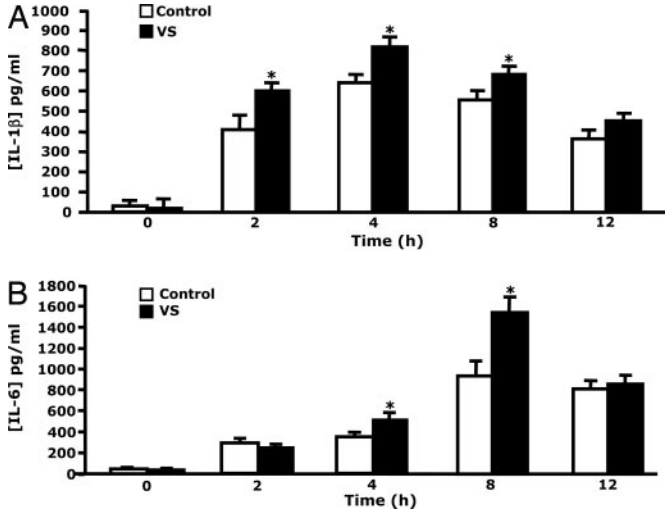

Fig. 2. VS increases peritoneal production of IL-1β and IL-6 in response to LPS. Mice (*db*/+) were treated with VS (0.5 mg/kg) or vehicle control (Control) for 7 days before i.p. injection of LPS (100 μg/kg). At the times indicated, peritoneal IL-1β (*A*) and IL-6 (*B*) were assayed by ELISA. Data in *A* represent means ± SEM; $n = 5$. *, $P < 0.05$ VS vs. control.

diabetic mice had significantly improved recovery from LPS-induced sickness at 8 h (31.7 ± 6.2% reduced vs. 52.7 ± 5.9% reduced) and 12 h (8.9 ± 7.7% reduced vs. 32.1 ± 9.3% reduced) than non-VS treated mice. To establish the impact of VS on neuroimmunity in *db*/+ mice, *db*/+ VS mice (0.5 mg/kg per day × 7 days) were challenged with or without i.p. LPS (100 μg/kg). A three-way ANOVA (VS × LPS × time) revealed significant VS × LPS × time interaction [F (5, 80) = 9.00, $P < 0.0001$] (Fig. 1*C*). VS markedly improved recuperation from sickness, shortening recovery time from 12 to 4 h without altering peak sickness at 2 h (91.7 ± 8.9% reduction vs. 95.5 ± 4.4% reduction). In addition, VS had no effect on mouse SE when LPS was not used as an immune challenge. Interestingly, when VS was administered as above but for only 12 h instead of 7 days, it did not impact SE (Fig. 6, which is published as supporting information on the PNAS web site). Taken together, these results indicate that *db*/*db* mice are resistant to the antiinflammatory actions of IGF-1 and that VS can act as an IGF-1 surrogate in *db*/+ and, importantly, *db*/*db* mice.

VS Increases Peritoneal Production of IL-1β and IL-6 in Response to LPS. LPS-induced expression of proinflammatory cytokines at the periphery is the initial step required for LPS-induced sickness (52). Therefore, given that VS-accelerated recovery from LPS-induced SE, it was anticipated that LPS-induced peritoneal levels of IL-1β, IL-6, and TNF-α would be reduced in VS-treated animals as compared with control mice. Fig. 2*A* shows that, when *db*/+ mice were administered VS (0.5 mg/kg per day) for 7 days and then challenged with LPS (100 μg/kg) as in Fig. 1*B*, peritoneal levels of IL-1β increased compared with non-VS-treated mice at 2 (599 ± 33 pg/ml vs. 409 ± 67 pg/ml), 4 (820 ± 37 pg/ml vs. 642 ± 41 pg/ml), and 8 h (681 ± 26 pg/ml vs. 557 ± 56 pg/ml) after LPS challenge. Likewise, LPS-induced IL-6 levels (Fig. 2*B*) were significantly increased at 4 (530 ± 52 pg/ml vs. 351 ± 48 pg/ml) and 8 h (1,540 ± 157 pg/ml vs. 940 ± 132 pg/ml) after LPS in VS animals compared with controls. VS had no impact on LPS-dependent TNF-α elaboration (Fig. 7*A*, which is published as supporting information on the PNAS web site), nor did it impact constitutive peritoneal IL-1β, IL-6, or TNF-α levels. Using a two-way ANOVA (VS × time), the main factors but not the interactions for IL-1β and TNF-α were significant for VS: F (1, 30) = 20.5, $P < 0.001$; time: F (1, 30) = 93.1, $P < 0.0001$. These data

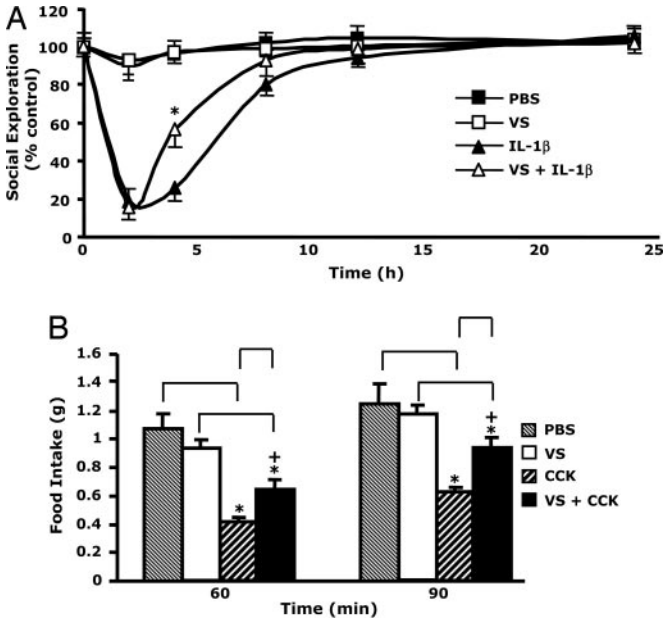

Fig. 3. Peripheral to central neural communication is blunted by VS. (*A*) Mice (*db*/+) were treated with VS (0.5 mg/kg) or vehicle control (PBS) for 7 days before i.p. injection of IL-1β (2 μg). SE was measured immediately before and at 2, 4, 8, 12, and 24 h after injection. Results are expressed as percentages of the baseline measurement and shown as means ± SEM; $n = 5$. *, $P < 0.05$ VS-IL-1β vs. PBS-IL-1β. (*B*) Mice (*db*/+) were treated with or without VS as above. Mice were then fasted for 16 h before administration of CCK-8 (CCK). Food was reintroduced, and food intake was measured at 60 and 90 min. Data represent means ± SEM; $n = 8$. * and +, $P < 0.05$.

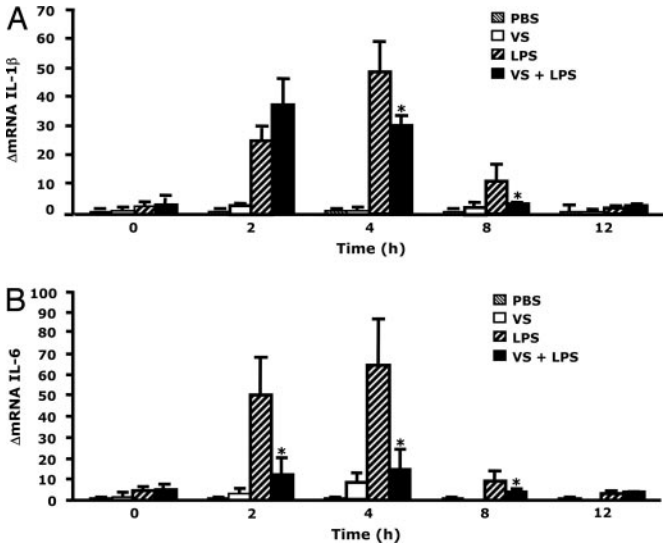

Fig. 4. VS inhibits LPS-dependent up-regulation of IL-1β and IL-6 in the brain. (*A* and *B*) Mice (*db*/+) were treated with VS (0.5 mg/kg) or vehicle control (PBS) for 7 days before i.p. injection of LPS (100 μg/kg). At the times indicated, total RNA was extracted from whole brains. Real-time RT-PCR was used to quantify IL-1β (*A*) and IL-6 (*B*) mRNAs relative to that of β actin. Results are expressed as relative change in mRNA expression (ΔmRNA) and are shown as means ± SEM; $n = 5$. *, $P < 0.05$ VS-LPS vs. PBS-LPS.

indicate that VS increased IL-1β levels in the peritoneal fluid independently of time. For IL-6 levels in the peritoneal fluid, the VS × time interaction was significant [F (4, 30) = 5.12, $P < 0.01$], indicating that the effect of VS varied according to time; they were significant only at 4 and 8 h after treatment. These results were confirmed in resident peritoneal macrophages isolated from 7-day VS (0.5 mg/kg per day) or saline-treated mice stimulated with LPS *ex vivo* (Fig. 7). Taken together, these data indicate that VS heightens responsiveness to LPS in the periphery.

Peripheral to Central Neural Communication Is Blunted by VS. Fig. 2 demonstrates that VS augments peritoneal macrophage responsivity to LPS and leads to increased LPS-dependent peritoneal IL-1β concentrations. Fig. 1 *B* and *C*, however, shows that VS-treated mice have enhanced recovery from sickness. Because IL-1β is thought to be the principal mediator of sickness behavior (46), the sickness response to peripherally administered IL-1β was examined in VS treated *db*/+ mice. As in Fig. 1*C*, mice were treated for 7 days with or without i.p. VS (0.5 mg/kg per day) and challenged with or without i.p. IL-1β (100 μg/kg). Fig. 3*A* shows the effect of VS and IL-1β on SE in *db*/+ mice. A three-way ANOVA (VS × IL-1β × time) revealed significant VS × IL-1β × time interaction [F (5, 80) = 13.14, $P < 0.0001$]. VS improved recovery from IL-1β-induced sickness at 4 h (43 ± 9.5% reduction vs. 74 ± 1.9% reduction). As in Fig. 1*B*, VS had no impact on peak sickness at 2 h (84.3 ± 2.2% reduction vs. 81.2 ± 0.9% reduction) or SE when IL-1β was not used as an immune challenge. To determine whether the brain itself had reduced sensitivity to LPS, *db*/+ mice were treated with VS as above and then administered 100 ng of LPS or vehicle control i.c.v. No effect of VS and LPS on SE when VS was injected i.c.v. was observed (Fig. 8, which is published as supporting information on the PNAS web site). Because immune-to-brain communication between the peritoneal cavity and CNS is mediated primarily by the vagus nerves (53), functionality of vagal afferents in VS-treated mice was examined by measuring CCK-8-dependent food intake suppression (54). As above, *db*/+ mice were treated for 7 days with or without VS then administered CCK-8 (16 μg/kg) or vehicle control after 16 h of starvation. Food intake was measured at 60 and 90 min after food reintroduction (Fig. 3*B*). Main factors but not the VS × CCK-8 × time or VS × time interaction were significant: VS, F (1, 32) = 60.74, $P < 0.0001$; time, F (2, 64) = 108.48, $P < 0.0001$, indicating that VS decreased feed intake independent of time. CCK-8 significantly reduced food intake at 60 (0.41 ± 0.03 g vs. 1.08 ± 0.1 g) and 90 min (0.63 ± 0.03 g vs. 1.25 ± 0.14 g). Importantly, VS inhibited the CCK-8-depressing action on food intake at 60 and 90 min to 0.65 ± 0.07 and 0.93 ± 0.08 g, respectively. VS had no effect on control animal food intake at either time point. Taken together, these results indicate that VS facilitates recovery from LPS-induced sickness behavior by altering vagally mediated communication from the periphery to the brain.

VS Inhibits LPS-Dependent Up-Regulation of IL-1β and IL-6 in the Brain. As shown in Fig. 3, VS blunts vagally transmitted information to the brain despite the proinflammatory effect VS has on peritoneal macrophages (Fig. 2). To understand how VS improves recovery from sickness, LPS-induced cytokine mRNA expression was examined in the brains of *db*/+ male mice treated for 7 days with or without i.p. VS (0.5 mg/kg per day) (Fig. 4). A three-way ANOVA (VS × LPS × time) revealed a significant VS × LPS × time interaction: IL-1β, F (4, 80) = 33.59, $P < 0.0001$; IL-6, F (4, 80) = 85.01, $P < 0.0001$; TNF-α, F (4, 80) = 2.54, $P < 0.05$. IL-1β mRNA up-regulation (Fig. 4*A*) was reduced in VS compared with control animals at 4 h (29.4 ± 4.2 vs. 49.1 ± 9.9 ΔmRNA) and 8 h (3.2 ± 0.5 vs. 11.2 ± 5.6 ΔmRNA). Interestingly, the LPS-induced IL-1β message was not significantly different in VS and control mice (36.5 ± 9.8 vs. 25.3 ± 4.4 ΔmRNA) at 2 h. Fig. 4*B* demonstrates that LPS-induced IL-6 mRNA was markedly different in VS and control mice at 2 h (11.5 ± 8.7 vs. 50.3 ± 17.6 ΔmRNA), 4 (14.4 ± 9.9 vs. 64.3 ± 21.8 ΔmRNA), and 8 h (2.3 ± 0.6 vs. 9.3 ± 3.7 ΔmRNA). VS had no impact on TNF-α message up-regulation in response to LPS (Fig. 9, which is published as supporting information on the PNAS web site). VS also did not increase

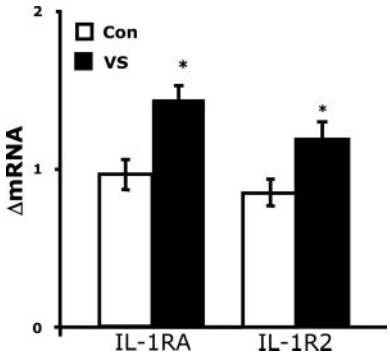

Fig. 5. VS up-regulates IL-1RA and IL-1R2 in the brain. Mice (*db*/+) were treated with VS (0.5 mg/kg) or vehicle control (PBS) for 7 days. Total RNA was extracted from whole brains. Real-time RT-PCR was used to quantify IL-1RA and IL-1R2 mRNAs relative to that of β actin. Results are expressed as relative change in mRNA expression (ΔmRNA) and shown as means ± SEM; $n = 5$. *, $P < 0.05$ VS vs. PBS.

constitutive (0 h) IL-1β, IL-6, or TNF-α mRNA in any of the brains examined. Taken together, these findings indicate that VS dampens LPS-dependent IL-1β mRNA expression in a manner reflecting the observed sickness (Fig. 1*B*), with peak expression unaffected but having an accelerated return to baseline. Additionally, the LPS-dependent IL-6 message was completely blocked by VS.

VS Up-Regulates the IL-1 Receptor Antagonist (IL-1RA) and the Type 2 IL-1 Receptor (IL-1R2) in the Brain. The counterregulation/modulation of IL-1β signaling outside of the cell is achieved by the specific IL-1 receptor antagonist, IL-1RA, and the decoy receptor, IL-1R2 (55, 56). To determine whether VS altered brain-based IL-1RA and IL-1R2 mRNA levels, VS treated (0.5 mg/kg per day × 7 days) *db*/+ male mice were examined (Fig. 5). A three-way ANOVA (VS × LPS × time) revealed a significant VS × LPS × time interaction: IL-1RA, F (4, 80) = 66.43, $P < 0.0001$; IL-1R2, F (4, 80) = 84.42, $P < 0.0001$. VS increased cerebral IL-1RA (1.42 ± 0.11 vs. 1.0 ± 0.07 ΔmRNA) and IL-1R2 (1.23 ± 0.06 vs. 0.95 ± 0.08 ΔmRNA) mRNA expression. Taken together, these findings indicate that VS increases the IL-1RA and IL-1R2 message in the brain.

Discussion

The present results show that pretreatment with VS attenuates LPS and IL-1β-induced sickness behavior despite enhanced cytokine production in the periphery. In contrast, IL-1β and IL-6 expression are decreased in the brain, whereas IL-1RA and IL-1R2 are enhanced. These data show that a micronutrient impacts vagal transmission of immune messaging from periphery to brain. We have shown that *db*/*db* mice exhibit impaired recovery from LPS-induced reductions in SE because of attenuated levels of IL-1RA in the brain (2). We have also shown that centrally administered IGF-1 can improve recovery from LPS-induced sickness in wild-type mice (49). Fig. 1*A* shows that IGF-1 administered peripherally improved recovery from LPS-induced sickness in wild-type but not in *db*/*db* mice. Previous studies by us (57–60) have shown that the antiinflammatory cytokines IL-1RA and IL-10 can down-regulate sickness, but these have all been administered i.c.v. Equally important is that *db*/*db* mice were resistant to the sickness-improving features of IGF-1 (Fig. 1*A*). This finding of functional IGF-1 resistance was surprising, in that the diabetic state appears to be significantly more associated with insulin resistance as opposed to IGF-1 resistance (61, 62). Furthermore, it was previously unclear whether physiologic IGF-1 resistance even existed in diabetes (63, 64).

Fig. 1 *B* and *C* shows that when VS was administered for 7 days, recovery from LPS-induced sickness in *db*/*db* and *db*/+ mice was dramatically improved. These data were unexpected, because VS has been shown to be a proinflammatory molecule (65) that enhances host susceptibility to infection (66). Importantly, enhanced macrophage activation by LPS would likely cause greater proinflammatory cytokine elaboration. Figs. 2 and 7 demonstrate that peritoneal and *ex vivo* macrophage-produced IL-1β and IL-6 in response to LPS was increased in VS-treated mice. Because IL-1β and IL-6 are the proinflammatory cytokines principally responsible for causing sickness behavior and fever (67–71), it was not anticipated that VS-treated mice would recover from sickness sooner. In addition, we have shown in IL-6 knockout animals that IL-6 synergizes with IL-1β to enhance LPS and/or IL-1β to augment sickness (47). Therefore, with increased LPS-induced peritoneal IL-1β and IL-6 levels, VS-treated mice would be expected to have more severe and prolonged sickness. This was not observed (Fig. 1 *B* and *C*). Furthermore, finding that VS-treated *db*/*db* mice recovered from sickness nearly 50% faster than sham-treated *db*/*db* mice (Fig. 1*B*) indicates that, despite peripheral resistance to IGF-1 (Fig. 1*A*), VS can mediate a neuroimmune antiinflammatory effect like IGF-1 but in diabetes. This finding is important, because it shows a potential therapy for diabetes-associated dysregulation of brain-immune signaling.

The neuroimmune modulating role of VS identified above requires VS to be administered in a chronic manner, because short-term VS exposure (12 h) had no effect on sickness (Fig. 6). The dose of VS chosen was identical to the oral dose of 0.5 mg/kg per day administered for 12 wk to weight-training athletes by Fawcett *et al.* (72), in which toxic effects of VS were not observed. To further explore how VS enhances recovery from sickness, IL-1β was administered i.p. to bypass the requirement of LPS to induce IL-1β (Fig. 3*A*). Improved recovery was seen in VS-treated animals, showing that VS-dependent recovery from sickness was not specific to the use of LPS. Interestingly, when VS mice were challenged with i.c.v. LPS, VS had no impact on peak sickness or on recovery from sickness (Fig. 9). Therefore, to determine how VS alters sickness, brain–immune communication was examined. Fig. 3*B* shows that VS blocked the satiety-inducing effect of CCK-8, likely by compromising the transmission of vagally mediated information from the periphery to the brain (53).

As we (73, 74) and others (75–77) have shown, the vagus nerve is essential for communicating peripheral sickness signals from the peritoneal cavity to the brain. Fig. 4 demonstrates that up-regulation of the brain IL-1β and IL-6 message in response to i.p. LPS was blunted in VS-treated mice. As in the peritoneum, TNF-α up-regulation was not significantly altered by VS (Fig. 9). This finding is important, because it indicates that VS was not just a neurotoxin that indiscriminately disabled immune-to-brain communication. IL-1β mRNA expression in VS mice paralleled the sickness behavior observed, because at 2 h (peak sickness), no significant difference was observed, whereas, at 4 and 8 h, the IL-1β message more rapidly approached baseline. Also of note was the marked suppressive effect VS had on LPS-dependent IL-6 mRNA up-regulation, suggesting that IL-6 has a much greater impact on sickness recovery than on peak sickness. To our knowledge, such dissociation between IL-1β and IL-6 has not been previously reported.

Fig. 5 demonstrates that VS increased basal IL-1RA and IL-1R2 mRNA in the brain by 50% and 30%, respectively. The biological activity of IL-1β is, in part, counterregulated through IL-1RA and IL-1R2. IL-1RA binds the active receptor (IL-1R1) with high affinity but does not initiate the intracellular signaling cascade (78). IL-1R2 acts as a competitive inhibitor that binds and sequesters IL-1β but lacks a functional intracellular Toll-IL-1 receptor domain (79). We have shown that i.c.v. administration of IL-1RA attenuates sickness induced by IL-1β (59), and immunoneutralization of IL-R2 potentiates IL-1β-induced anorexia (80). Diabetes appears to be a

subacute chronic inflammatory condition that is associated with a marked reduction in IL-1RA (2). We have shown that basal levels of IL-1RA are 14-fold reduced in the peritoneum of *db*/*db* mice compared with *db*/+ mice, and that IL-1RA and IL-1R2 fail to up-regulate in the brain of *db*/*db* mice in response to LPS (2). Therefore, strategies to increase IL-1 antagonists, like VS supplementation, in individuals with type 2 diabetes should prove advantageous in aiding in their recovery from sickness.

Conclusion

How VS improves recovery from sickness appears to be 3-fold, involving up-regulation of IL-1 antagonists, damping of brain-based proinflammatory cytokine up-regulation, and blunting of vagally mediated periphery to central communication. VS as a dietary supplement may have the real benefit of damping the duration of the sickness response and aiding an individual's return to the feeling of well being, as opposed to being a glucose-lowering agent of dubious reputation (6).

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