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Stress decreases, while central nucleus amygdala lesions increase, IL-8 and MIP-1α gene expression during tissue healing in non-human primates

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Abstract

Stress impairs healing and in part this effect is thought to be mediated by glucocorticoids. However, the brain systems that underlie the effects of stress on healing remain to be determined. Since the central nucleus of the amygdala (CeA) plays a role in mediating an individual's behavioral and physiological reactivity to stress, we investigated, in rhesus monkeys, whether selective lesions of the CeA altered the gene expression of chemokines (IL-8 and MIP-1α) that are associated with early dermal healing. We used rhesus monkeys because they provide an excellent animal model to investigate brain mechanisms relevant to human stress, anxiety, and psychopathology. Hypothalamic–pituitary–adrenal (HPA) activity was assessed in the monkeys prior to the wound healing experiment demonstrating that the CeA lesions reduce HPA activity. In the healing experiment, stress decreased IL-8 and MIP-1α gene expression in both CeA lesioned and non-lesioned animals. Conversely, the CeA lesions increased the tissue expression of IL-8 and MIP-1α mRNA prior to and after stress exposure. These results demonstrate that in primates the CeA is a key brain region involved in the regulation of processes associated with wound healing. Because of brain and behavioral similarities between rhesus monkeys and humans, these results are particularly relevant to understanding brain mechanisms that influence healing in humans.

Keywords: CeA; Chemokine; HPA axis; Rhesus monkey; Stress; Wound healing; Inflammation

1. Introduction

While adaptive, the stress response can also have deleterious effects (McEwen and Seeman, 1999). For example, studies in rodents and humans demonstrate that psychological stress significantly delays wound healing (Glaser et al., 1999; Marucha et al., 1998; Sheridan et al., 2004). In part, this effect is thought to be mediated by activation of the hypothalamic–pituitary–adrenal (HPA) axis. Studies in rodents and humans implicate corticosterone or cortisol in this process, as it has been shown that activation of the HPA axis in response to psychological stress can significantly delay wound healing (Kiecolt-Glaser et al., 1995; Marucha et al., 1998; Padgett et al., 1998) and suppress the function of important parameters of the immune system (Padgett et al., 1998). To understand the brain mechanisms that mediate the effects of stress on wound healing, we studied rhesus monkeys with bilateral lesions of the central nucleus of the amygdala (CeA). We used rhesus monkeys because they are similar to humans in brain structure and

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social behavior and therefore are an excellent model for exploring basic mechanisms underlying human fear, anxiety, and stress (Kalin and Shelton, 2003).

We hypothesized involvement of the amygdala in mediating the effects of stress on wound healing since numerous studies in rats, monkeys, and humans demonstrate the importance of the amygdala in mediating the stress response as well as associated fear and anxiety (Kalin et al., 2004; Phelps and LeDoux, 2005). The amygdala is a complex structure that consists of numerous nuclei, including the lateral and central nucleus. In processing responses to fear-related stimuli, the lateral nucleus of the amygdala receives information from other brain regions which via intraamygdaloid connections is conveyed to the CeA (Amaral et al., 1992; LeDoux, 1998). Through direct and indirect pathways, the CeA sends efferents to brain regions that mediate fear-related emotional, autonomic, and hormonal responses to psychological and physical stressors (Amaral et al., 1992). In prior work using site-specific neurotoxic lesions of the CeA in non-human primates we established the importance of the CeA in mediating trait-like anxiety and in the expression of fear-related behavioral and physiological responses, including HPA activation (Kalin et al., 2004). Therefore, in the present study, we used a subset of these animals to examine the role of CeA in mediating effects of stress on molecular changes associated with early wound healing. The effects of CeA lesions on HPA activity prior to this healing study were also assessed.

We focused our efforts on assessing the impact of the CeA lesions on the mRNA expression of two chemokines, interleukin [IL]-8 and macrophage inflammatory protein MIP-1α. Following tissue injury, these chemokines are expressed early in the inflammatory process (Holzheimer and Steinmetz, 2000; Werner and Grose, 2003), and are critical for the proper recruitment and activation of neutrophils and monocytes, respectively (DiPietro et al., 1998; Gillitzer and Goebeler, 2001; Godaly et al., 2001). MIP-1α highly correlates with macrophage numbers at the wound site (Werner and Grose, 2003), and IL-8 has been strongly associated with stress-mediated changes in wound tissue in humans (Glaser et al., 1999). Although pro-inflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α) have been shown to be important in wound inflammation (for review see Werner and Grose, 2003), these two pro-inflammatory chemokines were focused upon as they are chemotactic to the main infiltrating immune cells in the early response following wounding and correlate highly with other inflammatory markers (e.g., IL-1β) (Glaser et al., 1999; Sato et al., 1999; unpublished observations in humans).

Since stress impairs healing processes, we predicted that stress would decrease the gene expression of the chemokines in wounded tissue and that this effect would be reduced in the CeA-lesioned animals. Based on earlier work, we also expected that the CeA-lesioned animals would exhibit decreased HPA activity (Kalin et al., 2004).

2. Materials and methods

2.1. Experimental subjects and lesioning procedure

The subjects were 24 male rhesus monkeys (*Macaca mulatta*; 2.9–5.6 years of age). Sixteen animals served as non-lesioned controls and eight animals received bilateral ibotenic acid lesions of the CeA. These animals are a subset of CeA-lesioned animals from which other behavioral and physiological data have been reported (Kalin et al., 2004). Animal housing and all experimental procedures were in accordance with institutional guidelines. Lesion procedures were performed aseptically under anesthesia by delivering from 2 to 10 1 μl injections of ibotenic acid (1 mg ibotenic acid hydrate/100 μl of phosphate buffered saline) bilaterally into the CeA. The stereotaxic coordinates for the injections were defined for each monkey from their individual MRI (Kalin et al., 2004). To assess the extent of CeA damage, as well as damage to adjacent regions, the lesioned animals were euthanized using methods consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. The brains were fixed, removed, and coronally sectioned for Nissl staining with thionine and glial staining with an antibody to glial fibrillary acidic protein (Kalin et al., 2004). In the eight animals, the amount of bilateral CeA damage ranged from 52 to 98% (mean = 71%) (see Table 1). The lesions were highly specific with some damage occurring in surrounding regions including the medial nucleus and dorsally in the basal forebrain areas (for more detail see Kalin et al., 2004). The animals were lesioned on average 18 months prior to this study. To understand the effects of the lesions on the HPA axis, cortisol and ACTH were assessed 16.9 months prior to this study on two occasions separated by one week. Blood was sampled without anesthesia between 08:15 and 09:15 h. Animals were then exposed to 30 min of confinement stress after which blood was immediately resampled.

2.2. Wound, biopsy, and stress procedures

All tissue samples were obtained while the animals were anesthetized with ketamine HCl (15 mg/kg IM.) On day 1, between 08:15 and 09:30 h, a full thickness dermal wound (3.5 mm in diameter) was placed on the left or right scapular area (counterbalanced among subjects) of each animal's back using a sterile tissue punch. This collected tissue represented unwounded skin. To evaluate the molecular changes occurring with early healing, 6 h post-wounding the animals were anesthetized with ketamine and the initial wound was biopsied. Since healing occurs at the wound margin, a 6.0-mm dermal biopsy was taken from the tissue surrounding the previously wounded skin that encompassed the site of the first 3.5 mm wound. Animals were allowed to recover from anesthesia for 2 h, and then were removed from their home cage and confined for 1 h in a transport cage. This procedure is stressful and results in marked activation of the HPA axis (Kalin and Shelton, 1984; Kalin et al., 2004). The same wounding and biopsy procedures were repeated on days 2 and 3, with the exception that on day 2 the new wound was placed in the midline of each animal's back, and on day 3 the new wound was placed in the scapular area contralateral to the day 1 wound site. Animals were again exposed to

Table 1
Percent of CeA destruction

% Destruction			
Subject	Left CeA	Right CeA	Total CeA
1	99	97	98
2	90	78	84
3	55	89	72
4	59	77	68
5	64	71	68
6	83	52	67
7	67	47	57
8	51	52	52
Mean ± SE	71.1 ± 6	70.4 ± 7	70.7 ± 5

the confinement stress on day 2 but not on day 3. Thus, day 1 tissue samples served as the baseline, since the animals were exposed to minimal stress. In contrast, day 2 and 3 samples reflected the cumulative effects of stress from previous wounding and biopsy procedures along with the experimentally induced confinement stress (Kalin et al., 2001, 2004).

2.3. Immune measurements

Both unwounded and wounded tissue samples were stored in 1 ml Trizol at −70 °C. mRNA was isolated and cDNA was synthesized as previously described (Gajendrareddy et al., 2005). The levels of IL-8 and MIP-1α mRNA, standardized to pyruvate dehydrogenase (PDH) mRNA levels, were determined using real time PCR (ABI Prism 7700-sequence detection system) following the manufacturer's protocol (Applied Biosystems, Foster City CA). Chemokine gene expression was expressed relative to PDH gene expression to eliminate differences in tissue sample volumes and processing yields.

2.4. ACTH and cortisol assays

Plasma was immediately separated from whole blood by centrifugation at 4 °C and frozen at −70 °C until assayed. Cortisol was measured in plasma samples using an enzyme immunoassay kit (Diagnostic Systems Laboratories, Webster, TX). The intraassay CV% was 6.1% and interassay CV% was 6.3%. The detection limit for this assay was 0.125 ng. ACTH was measured using a radioimmunoassay (Nichols Institute Diagnostics, San Clemente, CA). The intraassay CV% was 2.2% and interassay CV% was 7.2%. The detection limit of the assay was 1.0 pg.

2.5. Data analysis

Values for IL-8 and MIP-1α were standardized to PDH values. Because these values and those for ACTH and cortisol were not normally distributed, log transformations were performed. Separate repeated measures ANOVAs were used to analyze each mRNA. Group (CeA lesion vs. control) was a between-subjects factor, whereas tissue-type (unwounded vs. wounded) and day (day 1-baseline; days 2 and 3-stress) were within-subject factors. Violations of sphericity were corrected using the Greenhouse-Geisser method. A small number of missing mRNA values (6 data points out of a possible 360) were replaced with group means. For each mRNA assessment, one different control subject was determined to be an outlier (>3 SDs from the group mean) and was excluded. Therefore, the data analyzed for each chemokine was from 15 control and 8 CeA-lesioned subjects.

3. Results

3.1. Effects of CeA lesions on basal and stress-induced HPA activity

To understand the extent to which the CeA lesions altered the tone of the HPA axis, hormonal data collected prior to the healing study were analyzed. A repeated measures ANOVA [with group (CeA lesion vs. control), condition (baseline vs. confinement stress), and test (test 1 vs. test 2) as factors] revealed a main effect of condition on ACTH concentrations [$F(1,22) = 159.61$, $p < .0001$] such that stress resulted in an increase from a mean ± SE baseline of 112.6 ± 9.3 to 292.9 ± 17.2 pg/ml. The CeA lesions resulted in lower ACTH concentrations across the baseline and stress sampling periods (Fig. 1A). The mean ± SE ACTH concentration for the CeA lesion group was 162.5 ± 22.5 pg/ml, whereas the mean ACTH concentration for the control group was 222.9 ± 16.2 [$F(1,22) = 5.17$, $p < .04$]. Cortisol concentrations were also affected by the confinement stress [$F(1,22) = 124.31$, $p < .0001$]. The mean ± SE baseline cortisol concentration was 35.9 ± 2.2 μg/dl compared to the mean stress concentration of 50.7 ± 2.3 μg/dl. Across both baseline and stress conditions, the CeA lesions resulted in non-significantly lower cortisol concentrations (control group = 46.0 ± 2.2; CeA lesion group = 37.9 ± 2.8) [$F(1,22) = 1.77$, $p = .197$] (Fig. 1B).

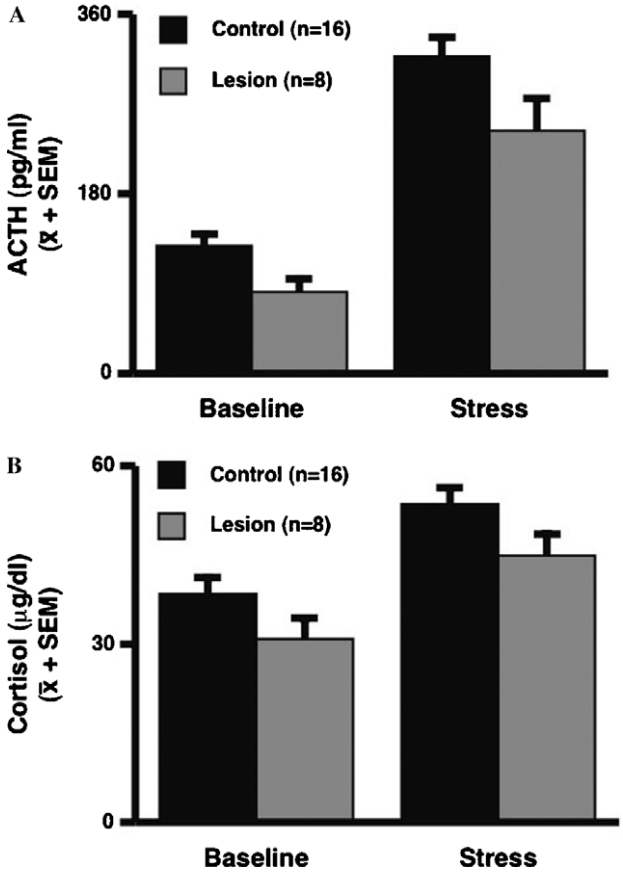

Fig. 1. Data collected prior to this healing study showed that CeA lesions (□; $n = 8$) compared to controls (■; $n = 16$) resulted in (A) significantly lowered ACTH concentrations ($p < .04$) and (B) non-significantly lower cortisol concentrations ($p < .20$) across both baseline and stress conditions. Values represent the mean and standard error of two baseline and two stress samples.

3.2. Effects of CeA lesions on the tissue mRNA expression of IL-8 and MIP-1α

Analysis of the chemokine data revealed a main effect of wounding for IL-8 [$F(1,21) = 199.94$, $p < .0001$] (Fig. 2A) and MIP-1α [$F(1,21) = 60.79$, $p < .0001$] (Fig. 2B), such that mRNA levels for both chemokines increased after wounding. A close to significant main effect of day was found for IL-8 [$F(2,21) = 2.74$, $p < .08$] and a significant main effect was found for MIP-1α [$F(2,21) = 5.69$, $p < .008$]. These effects were characterized by decreases in mRNA concentrations from day 1 to days 2 and 3 associated with the cumulative stress exposure (Fig. 2). There was also a main effect of group such that the CeA lesions resulted in a

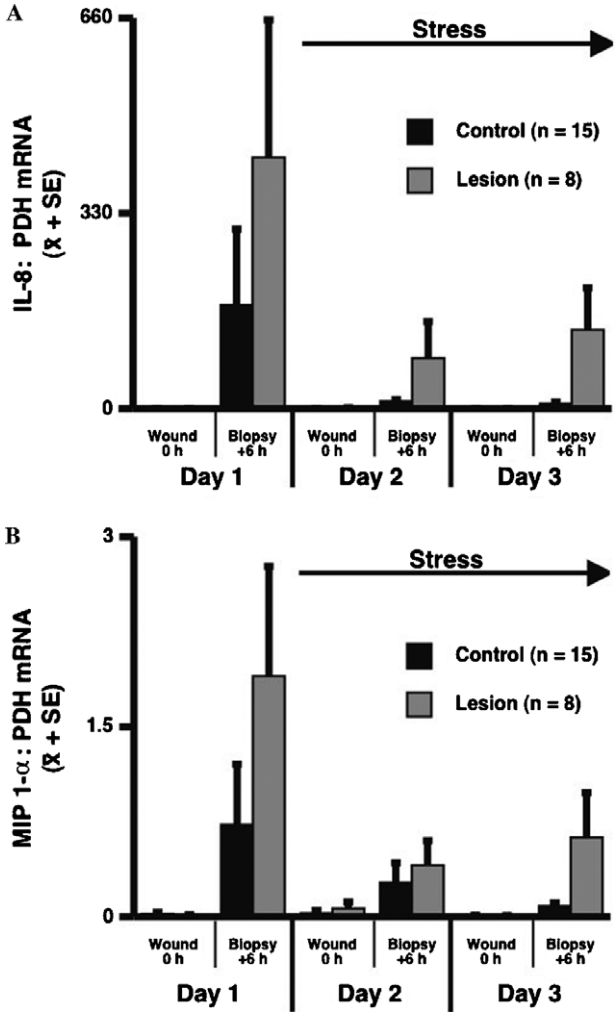

Fig. 2. IL-8 and MIP-1α mRNA were standardized to PDH mRNA values. On each day, initial wounding occurred at 08:15 h and the biopsy of the wound was taken 6 h later. A main effect of wounding was found such that on all days the expression of (A) IL-8 mRNA ($p < .0001$) and (B) MIP-1α mRNA ($p < .0001$) was higher in the biopsy samples, reflecting the healing process, compared to those from the initial wounds. In addition, for both chemokine mRNAs, day 1 levels were higher compared to those after stress exposure on days 2 and 3 (IL-8, $p < .08$; MIP-1α, $p < .008$). Finally, a significant main effect of lesion was observed for IL-8 mRNA expression ($p < .0006$) and was close to reaching significance for MIP-1α mRNA expression ($p < .08$). On all days, mRNA expression was higher for CeA-lesioned animals compared to controls.

significant increase in the expression of IL-8 mRNA across all days [$F(1,21) = 16.18$, $p < .0006$]. A close to significant effect of the lesions was also found for MIP-1α [$F(1,21) = 3.43$, $p < .08$]. In addition, the day × wounding interaction approached significance for IL-8 [$F(2,42) = 2.86$, $p < .08$] and a significant interaction was found for MIP-1α [$F(2,42) = 10.13$, $p < .001$]. The pattern of change for both chemokines in the biopsy wound was increased on day 1 and decreased over days 2 and 3. In unwounded tissue, the chemokines were not elevated on day 1 and did not change on days 2 and 3. There were also significant lesion × wounding interactions for IL-8 [$F(1,21) = 14.53$, $p < .001$] and MIP-1α [$F(1,21) = 5.15$, $p < .04$]. Compared to controls, the CeA-lesioned animals had significantly higher levels of both IL-8 and MIP-1α mRNA in wounded tissue across all days. The lesion × day interactions for IL-8 and MIP-1α were not significant. The lesion × wounding × day interaction was not significant for IL-8 mRNA [$F(2,42) = 1.42$, $p = .2536$]. The 3-way interaction for MIP-1α demonstrated a trend towards significance [$F(2,42) = 2.75$, $p = .0910$]. As can be seen in Fig. 2 the chemokines measured from the biopsies of the wounded tissue on day 1 are higher for the lesion group and were also higher for the lesion group as compared to controls on subsequent days.

4. Discussion

While established in rodents and humans (Kiecolt-Glaser et al., 1995; Marucha et al., 1998; Padgett et al., 1998; Rojas et al., 2002; Sheridan et al., 2004), this is the first demonstration in non-human primates that stress alters the molecular mechanisms associated with wound healing. We found that the cumulative stress associated with the experiment blunted increases in IL-8 and MIP-1α expression during the early phases of wound healing. Increased gene expression of IL-8 and MIP-1α after wounding likely promotes healing as expression of these chemokines is related to both recruitment and increased activation of neutrophils and monocytes (Baggiolini et al., 1995). We focused our investigations on IL-8 and MIP-1α because pro-inflammatory cytokines following wounding are (1) expressed in cascades; (2) similarly regulated by stress hormones; and (3) correlate closely with both these chemokines (Glaser et al., 1999; Sato et al., 1999; unpublished observations in humans). Thus, we expect other pro-inflammatory cytokines to have similar relationships to both stress and CeA lesions as those observed in the current study.

The mechanisms that underlie the stress-induced reductions in IL-8 and MIP-1α are thought to be mediated in part by HPA activity. In rodents, increased circulating levels of corticosterone appear to be important in impeding the healing process (Padgett et al., 1998; Sheridan et al., 2004). In humans, increased cortisol concentrations have been associated with decreased expression of IL-1 and IL-8 at the wounding site early in the healing process (Glaser et al., 1999). When we tested these same animals months before the current healing study, we found that 30 min of confinement stress robustly increased ACTH and cortisol concentrations in both groups of animals. Thus, we expect that the 60 min of confinement used during this study, plus any cumulative effects of additional stressors (e.g., anesthesia, wounding), resulted in similar or greater HPA activation.

We hypothesized involvement of the amygdala in mediating the effects of stress on healing, since it is a brain region that is a key component of the neural circuitry that mediates the expression of fear-related behavioral and physiological responses, as well as anxiety (Davis and Whalen, 2001; LeDoux, 1998). We specifically

examined the role of the CeA, because it projects to hypothalamic and brain stem regions involved in the regulation of the HPA and autonomic systems (Davis and Whalen, 2001; LeDoux, 1998). The data demonstrate that, compared to controls, the CeA-lesioned animals had increased IL-8 and MIP-1α gene expression and this effect was only observed in wounded tissue. This indicates that the CeA lesions selectively affected chemokine mRNA levels in response to wounding (i.e., immune activation). However, the CeA lesions did not influence the apparent effect of stress on wound healing. This is supported by the finding that stress reduced chemokine gene expression in both the control and CeA-lesioned groups. Overall, CeA lesions appear to increase, and stress to decrease, chemokine gene expression in wounded tissue, independent of one another. Possibly stress and the CeA act independently on the same pathway (e.g., HPA axis). However, CeA lesions have been shown to attenuate HPA responses to surgery stress (Shavit et al., 2005), indicating these factors can interact along the HPA pathway. This suggests that a second pathway of chemokine activation might be involved in the present model (e.g., sympathetic nervous system).

It is of interest that increased chemokine expression was detected as early as day 1 in the CeA-lesioned animals. Consistent with this was the observation that ACTH concentrations were lower in the CeA-lesioned group compared to the control group when assessed prior to this experiment. Cortisol concentrations were also lower in the CeA group but the magnitude of this effect did not attain statistical significance. Therefore, it is possible that the CeA lesions affected healing by persistently reducing the overall tone of the HPA system. In an earlier report, we presented data from a group of monkeys that included these animals, demonstrating that the CeA lesions blunted fear and anxiety responses as well as pituitary–adrenal activity (Kalin et al., 2004).

These findings support the use of the rhesus monkey as a model to link rodent to human studies investigating the influences of brain function and psychological stress on wound healing. Furthermore, the findings demonstrate that selective lesions of the CeA have effects opposite to those of stress and increase IL-8 and MIP-1α gene expression, possibly through decreased activation of the HPA system. These findings provide insights into the brain systems involved in modulating wound healing and are the first to implicate a role for the amygdala.

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