Psychoneuroendocrinology 99 (2019) 191–195

Contents lists available at ScienceDirect

Psychoneuroendocrinology

journal homepage: www.elsevier.com/locate/psyneuen

Circulating epinephrine is not required for chronic stress to enhance metastasis

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ARTICLE INFO

Keywords:
Epinephrine
Norepinephrine
Cancer
Stress-enhanced metastasis
Splanchnic nerve
Inflammatory reflex

ABSTRACT

Signaling through β-adrenergic receptors drives cancer progression and β-blockers are being evaluated as a novel therapeutic strategy to prevent metastasis. Orthotopic mouse models of breast cancer show that β-adrenergic signaling induced by chronic stress accelerates metastasis, and that β_2-adrenergic receptors on tumor cells are critical for this. Endogenous catecholamines are released during chronic stress: norepinephrine from the adrenal medulla and sympathetic nerves, and epinephrine from the adrenal medulla. β_2-adrenergic receptors are much more sensitive to epinephrine than to norepinephrine. To determine if epinephrine is necessary in the effects of stress on cancer progression, we used a denervation strategy to eliminate circulating epinephrine, and quantified the effect on metastasis. Using both human xenograft and immune-intact murine models of breast cancer, we show that circulating epinephrine is dispensable for the effects of chronic stress on cancer progression. Measured levels of circulating norepinephrine were sufficiently low that they were unlikely to influence β_2-adrenergic signaling, suggesting a possible role for norepinephrine release from sympathetic nerve terminals.

1. Introduction

Beta-adrenergic receptor (βAR) signaling has been identified as a target to modulate cancer progression. Preclinical studies in many types of cancer found that βAR signaling drives cancer progression, and identified molecular and cellular mechanisms (Thaker et al., 2006; Nissen et al., 2018; Le et al., 2016; Kim-Fuchs et al., 2014; Lamkin et al., 2012; Sloan et al., 2010; Kim et al., 2016). These studies discovered that βAR signaling in tumor cells drives tumor cell invasion by enhancing formation of invadopodia, resulting in increased metastasis *in vivo* (Chang et al., 2016; Pon et al., 2016; Kim et al., 2016; Creed et al., 2015). Stromal cells in the tumor microenvironment also are responsive to βAR signaling. Both innate and adaptive immune cells express β_2AR (Nissen et al., 2018). βAR signaling in tumor-associated macrophages remodels vasculature to increase routes for tumor cell dissemination (Le et al., 2016; Sloan et al., 2010; Thaker et al., 2006), while βAR signaling in CD8+ cytotoxic T cells impairs anti-tumor immunity, which accelerates cancer and impairs immunotherapy (Bucsek et al., 2017; Kokolus et al., 2018; Nissen et al., 2018).

As a consequence of these findings, β-blockers are being evaluated as a novel strategy to slow cancer progression. Epidemiological studies have shown an association between β-blocker use and reduced metastasis (Watkins et al., 2015; Choi et al., 2014; Melhem-Bertrandt et al., 2011; Powe et al., 2010; Botteri et al., 2013; De Giorgi et al., 2013),

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https://doi.org/10.1016/j.psyneuen.2018.09.012
Received 5 July 2018; Received in revised form 11 September 2018; Accepted 11 September 2018

although not in all cohorts (Sorensen et al., 2013), and two prospective clinical studies have demonstrated that β-blockers reduce biomarkers of metastasis and improve survival (Shaashua et al., 2017; De Giorgi et al., 2018). A number of ongoing clinical studies are now evaluating β-blocker use in the perioperative period to prevent cancer recurrence after tumor-resection surgery (Hiller et al., 2017; Horowitz et al., 2015).

Signaling through βAR is induced by endogenous catecholamines; epinephrine and norepinephrine. The adrenal medulla is the main source of epinephrine (Esler et al., 1988, 1990), with neurotransmitter release tightly controlled by the splanchnic nerve (de Diego et al., 2008). The main source of norepinephrine is nerve terminals of the post-ganglionic sympathetic nervous system (SNS) that are present in peripheral organs including the breast (Eriksson et al., 1996; Furlan et al., 2016). A number of studies have demonstrated a critical role for signalling through the β_2AR receptor subtype in the effects of stress on cancer progression (Chang et al., 2016; Nissen et al., 2018; Thaker et al., 2006). β_2AR binds epinephrine with approximately 10-fold greater affinity than norepinephrine (pK_D = 6.13 vs. 5.41) (Baker, 2010). Therefore, to investigate the necessity of circulating epinephrine in the effect of stress on cancer progression, we used a denervation strategy to block the supply of epinephrine from the adrenal medulla into circulation, and examined the impact on breast cancer metastasis.

2. Methods

2.1. Animals

Female BALB/c *nu/nu* (Animal Resources Centre, Western Australia) and BALB/c mice (Monash University, Australia) were housed in a temperature and humidity controlled environment with a 12/12-h dark-light cycle. Food and water were available *ad libitum*. Mice were acquired at 6 weeks old and were given 1–2 weeks to acclimate before experimentation commenced with denervation surgery. Surgery was conducted from 7–8 weeks of age for studies in BALB/c mice, and at 8–9 weeks for studies in BALB/c *nu/nu* mice. All procedures involving mice were carried out under protocols approved by the Institutional Animal Ethics Committee and in accordance with National Health and Medical Research Council guidelines.

2.2. Denervation

The greater splanchnic nerves were exposed retroperitoneally on each side, as described previously for rats (Martelli et al., 2014a). The nerves were either cut bilaterally just beneath the diaphragm (Denervation group) or left intact (Sham group). Specifically, surgical access to the right nerve was made through a right flank incision (1–1.5 cm) below the ribs but above the pelvis, close to the back muscles. Incision of the thin muscle sheet next to the back muscles revealed the retroperitoneal space (including the kidney, adrenal gland and perirenal fat), which was opened with blunt separation and retraction while keeping the peritoneum intact. The adrenal gland was pulled downwards with a cotton bud to expose the splanchnic nerve, which was separated with fine forceps and then cut. The central branch was peeled away to eliminate the possibility of nerve regeneration. The left splanchnic nerve was cut by the mirror image procedure through a second incision. The incisions were closed by suturing and mice allowed to heal for four days prior to starting restraint stress. Surgery was performed under isoflurane anaesthesia (2% in oxygen).

2.3. Cell culture

The human metastatic breast cancer cell line MDA-MB-231^{HM} (Le et al., 2016) was cultured in DMEM-GlutaMAX media (Life Technologies), and the 66cl4 luminal B mammary adenocarcinoma cell line (Sloan et al., 2010) was cultured in αMEM-GlutaMAX media (Life Technologies). All media was supplemented with 10% fetal bovine serum (Life Technologies). All cells were maintained at 37 °C and 5% CO_2 and mycoplasma tested before injection using primers as described (Uphoff and Drexler, 2002). Cell line identities were confirmed by short tandem repeat profiling (Cellbank, Australia). All cells have been stably transduced with a non-replicating lentiviral construct containing luc2 (codon optimised luciferase) under control of the ubiquitin C promoter to allow *in vivo* monitoring (see below) with either mCherry or eGFP fluorescent protein for further analysis.

2.4. Animal breast cancer models

Either 2×10^5 MDA-MB-231^{HM} cells or 1×10^5 66cl4 cells in 20 µL PBS (Invitrogen) were injected into the 4th inguinal mammary fat pad of anesthetized (3% isoflurane) BALB/c *nu/nu* or BALB/c mice respectively. Primary tumor growth was monitored by digital caliper twice a week and volume calculated using the formula: (length × width^2)/2. Further, growth and metastasis of luciferase-tagged tumor cells were tracked non-invasively in mice with bioluminescence with Living Image Software on an IVIS Lumina II (Perkin Elmer) imaging system after mice received 150 mg/kg D-luciferin (Choice analytical) in 100 µL PBS via the tail vein. During the experiment, blood was collected at various time points in vials containing EGTA (Sigma-Aldrich) for catecholamine analysis. Plasma was collected after centrifugation at 1000*g*, 4 °C, and immediately snap frozen in liquid nitrogen.

2.5. Physiological activation of the SNS stress response

To physiologically activate neural signaling, mice were placed in a restraint apparatus for two hours per day for 21 consecutive days, starting five days prior to tumor cell injection (Thaker et al., 2006; Sloan et al., 2010; Le et al., 2016). This protocol has been previously used to demonstrate regulation of metastasis by stress in mouse models of breast cancer and other cancers (Le et al., 2016; Thaker et al., 2006; Kim-Fuchs et al., 2014; Sloan et al., 2010). Briefly, each mouse was placed into a well-ventilated compartment, and the space in each compartment was adjusted according to the length of each individual mouse so mice were maintained in a confined space (final dimensions: 30 × 28 × 40 mm).

2.6. Catecholamine analysis

Blood was collected from the submandibular vein four days after splanchnic denervation and again immediately after the third session of daily restraint. Blood was collected into chilled tubes containing EGTA and reduced glutathione, and plasma for catecholamine analysis was immediately separated by centrifugation at 4 °C and snap frozen in liquid nitrogen without delay. Samples were stored at −80 °C and analyzed by HPLC within 35 days of sample collection. Sampling, processing and storage of plasma according to these methods is associated with little change in catecholamines and metabolites (Lambert et al., 1991; Venneri and Del Rio, 2004). Catecholamines were extracted from plasma with alumina adsorption, separated by high-performance liquid chromatography, and quantified by coulometric detection according to previously described methods (Lambert and Jonsdottir, 1998).

2.7. Statistical analysis

All data are presented as means ± SE. Statistical differences in individual catecholamines were determined using Mann–Whitney Tests. Longitudinal analyses for primary tumor growth and metastasis were determined using Repeated Measures Analyses of Variance (ANOVA). Post-hoc analyses were conducted using Tukey's multiple comparison test. GraphPad Prism software, version 7 was used for data presentation and analysis.

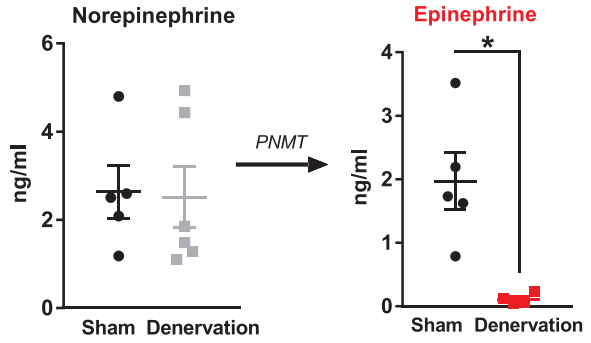

Fig. 1. Splanchnic denervation abrogates circulating epinephrine levels. Catecholamines were quantified in plasma by HPLC 4 days after denervation surgery. PNMT: Phenylethanolamine *N*-methyltransferase; $^{*}p < 0.05$.

3. Results

Action potentials in pre-ganglionic nerve fibers regulate release of epinephrine from the adrenal medulla. Therefore, cutting the splanchnic nerve prevents epinephrine release from the adrenal medulla. To determine if bilateral splanchic denervation was effective, we used HPLC to quantify the levels of circulating catecholamines in plasma of mice 4 days after denervation surgery. Denervation of the splanchnic nerves abrogated circulating epinephrine, reducing it by 20-fold compared to sham-operated controls (median sham vs denervated: 1732 vs. 89 pg/ml, $U = 0$, $p < 0.05$) but did not affect plasma norepinephrine ($p > 0.05$) (Fig. 1).

Under conditions of stress or fear, there is a surge of catecholamine release from the adrenal medulla and SNS nerves, which mobilizes the body for the 'fight-or-flight' response. To investigate if splanchic denervation eliminated the circulating epinephrine response to stress, plasma catecholamines were also assessed after induction of chronic stress by repeated daily restraint. Stress increased plasma epinephrine in sham-operated mice by 2.2-fold (1.73 vs. 3.72 ng/ml, $U = 0$, $p < 0.05$), but had no effect on circulating epinephrine in denervated mice (0.09 vs 0.11 ng/ml, $p > 0.05$) (Fig. 2A). In contrast, stress increased circulating norepinephrine levels in both sham-operated and denervated mice compared to baseline (1.6-4-fold, U = 20, $p < 0.05$) (Fig. 2B). There was no difference in stress-induced norepinephrine concentrations between denervated and sham-treated mice. To ensure that these findings were not confounded by metabolism by monoaminoxidase we assessed plasma dihydroxyphenylglycol (DHPG) concentration. DHPG was detected but levels were not affected by denervation or restraint stress ($p > 0.05$) (Supplementary Fig. 1). Collectively, these findings show that splanchnic denervation blocks baseline epinephrine release under resting conditions and prevents physiological elevation of epinephrine in response to stress.

Chronic stress acts through βAR signaling to enhance tumor growth

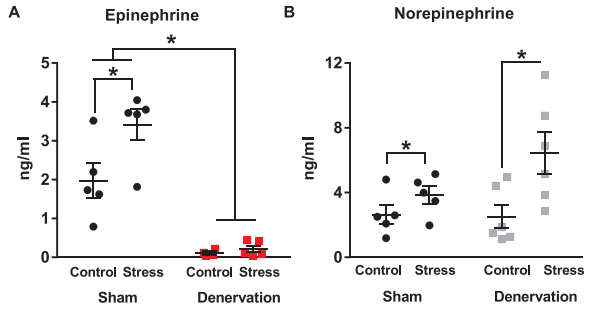

Fig. 2. Effect of stress on circulating norepinephrine and epinephrine levels. Catecholamines were quantified in plasma from denervated mice and sham-operated mice after three days of repeated daily restraint or home-cage conditions. $^{*}p < 0.05$.

and metastasis in mouse models of breast cancer (Le et al., 2016; Chang et al., 2016). To determine the necessity of plasma epinephrine in cancer progression, we investigated the effect of stress on metastasis in denervated mice. Human MDA-MB-231^{HM} breast cancer cells were injected into the mammary fatpad of mice after splanchnic or sham surgery. Mice were then exposed to chronic stress or control conditions during tumor development, as previously described (Le et al., 2016; Sloan et al., 2010). In mice with intact splanchnic nerves, stress enhanced primary tumor growth (Fig. 3A) and metastasis (Fig. 3B), consistent with previous findings (Le et al., 2016; Chang et al., 2016). Elimination of plasma epinephrine by splanchnic nerve denervation had no effect on stress-enhanced primary tumor growth or metastasis, indicating that circulating epinephrine is not necessary for breast cancer progression (Fig. 3).

Increasing evidence shows the importance of the adaptive immune response in regulating cancer progression (de Visser et al., 2006). We recently discovered that βAR signaling regulates CD8+ T cells to control anti-tumor immunity (Nissen et al., 2018). As the MDA-MB-231^{HM} cell-line requires assessment in nude mice, which lack functional T cells (Fig. 3), we evaluated if epinephrine contributes to cancer progression in mice with intact and functional T cells. 66cl4 cells express βAR and chronic stress drives metastasis in this model (Sloan et al., 2010; Pon et al., 2016). Balb/c mice were injected with 66cl4 tumor cells, which are syngenic in this immunocompetent mouse strain. Denervation had no effect on primary tumor growth or metastasis (Fig. 4A, B), confirming that circulating epinephrine is dispensable for breast cancer progression under stress conditions.

4. Discussion

Despite a wealth of evidence that neural signaling through β-adrenergic receptors drives breast cancer progression (Sloan et al., 2010; Le et al., 2016; Chang et al., 2016; Creed et al., 2015; Kim-Fuchs et al., 2014; Lamkin et al., 2012) the endogenous catecholaminergic neurotransmitter(s) responsible for activating βAR and the source of neurotransmitter release are unknown. Here we use denervation of the splanchnic nerve to selectively deplete plasma epinephrine, and demonstrate for the first time that circulating epinephrine is not required for neural regulation of breast cancer.

The findings suggest that it is unlikely that plasma catecholamines drive cancer progression. Antagonist studies using β-blockers have demonstrated that neural signaling promotes metastasis by activating βAR that are present on tumor cells (Chang et al., 2016; Creed et al., 2015; Sood et al., 2010) and on cells in the tumor microenvironment (Le et al., 2016; Nissen et al., 2018; Sloan et al., 2010). In particular, β_2AR on tumor cells and immune cells are important for the effect of stress on breast cancer progression (Chang et al., 2016; Le et al., 2016; Nissen et al., 2018). Epinephrine binds preferentially to β_2AR (circa 10-fold selectivity for β_2AR over β_1AR) and has a higher affinity for this receptor ($pK_D = 6.13$) than norepinephrine ($pK_D = 5.41$) (Baker, 2010). We found however, that the epinephrine levels available in the plasma of mice with intact splanchnic nerves were low (2–4 ng/ml, i.e., circa 10–20 nM), which would occupy only a very small fraction of the β_2AR on target cells. As such, the low occupancy of β_2AR achieved by circulating epinephrine means that any resulting signaling will depend on the agonist efficacy of epinephrine, and the signaling amplification and receptor expression level of the target cells. The fact that circulating epinephrine is not required for neural regulation of cancer progression strongly suggests that signaling efficiency and β_2AR expression in the relevant target cells is not sufficiently high for plasma epinephrine to have a major influence on their intracellular signaling. Even under stress conditions, norepinephrine in plasma only reached nanomolar levels (mean: 4 ng/ml, i.e., circa 20 nM; Fig. 2), suggesting that it is most unlikely that circulating norepinephrine (which has a similar agonist efficacy to epinephrine at β_2AR; (Baker, 2010)) impacted βAR signaling sufficiently to drive cancer progression.

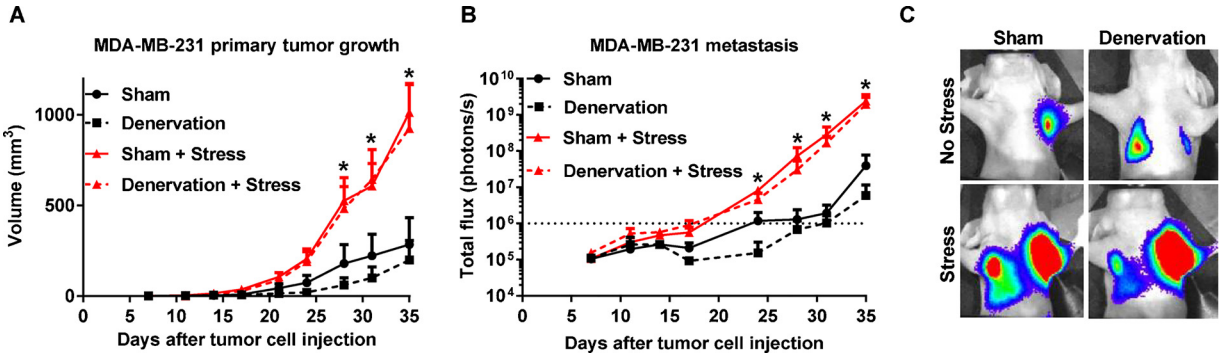

Fig. 3. Splanchic denervation has no impact on stress-enhanced cancer growth or metastasis in MDA-MB-231^{HM} breast cancers. Mice with bilateral splanchnic denervation or sham-operated mice were injected with MDA-MB-231^{HM} cells into the 4th left mammary fatpad. A. Primary tumor growth was monitored by caliper. B. Development of distant metastasis was quantified by longitudinal non-invasive bioluminescence imaging. C. Representative in vivo imaging of metastasis in lymph node and lung on day 35 of tumor growth.

These findings suggest that a likely source of neurotransmitter to drive breast cancer progression is the local release of norepinephrine from SNS nerve terminals. The normal breast (or mammary gland) is innervated (Eriksson et al., 1996) and catecholaminergic nerve fibers have been detected in both human tumours and mouse models of cancer (Szpunar et al., 2016; Cole et al., 2010; Allen et al., 2018). There is some evidence that breast tumor cells induce neo-neurogenesis through nerve growth factor-mediated mechanism (Boilly et al., 2017). Notably, social isolation has been linked to elevated levels of norepinephrine in tumours of ovarian cancer patients, consistent with the idea that chronic stress impacts neural signalling in cancers (Lutgendorf et al., 2011). Additionally, immune cells that are recruited to tumors and influence tumour progression may be exposed to neurotransmitters outside the primary tumor. For example, myeloid cells may be exposed to neurotransmitters in the bone marrow (Hanoun et al., 2015).There is growing understanding that SNS neural signaling in the bone marrow modulates immune function in ways that impact cancer progression (Maryanovich et al., 2018; Ben-Shaanan et al., 2018; Sloan et al., 2010; Le et al., 2016).

The finding that splanchnic denervation did not alter breast cancer progression is consistent with high levels of inflammation in aggressive cancer (de Visser et al., 2006). We recently characterised the critical role of the splanchnic nerve in the inflammatory reflex, which limits the acute inflammatory response (Martelli et al., 2014a, 2016). We found that lesioning the splanchnic nerve releases the brakes on inflammation, resulting in significant amplification of pro-inflammatory cytokine levels in response to an immune challenge (Martelli et al., 2014a,b). Inflammation is known to drive cancer progression, and neural signaling through βAR controls the phenotype of macrophages and their recruitment to tumors to accelerate metastasis (Sloan et al., 2010; Le et al., 2016; de Visser et al., 2006; Lamkin et al., 2016). This raises the possibility of opposing actions of epinephrine – regulated by the splanchnic nerve to control inflammation – and norepinephrine – released from local nerve terminals to drive tumor-associated inflammation and therefore cancer progression. This possibility is not directly tested here and will be followed up in future studies.

In sum, these findings show that circulating epinephrine is not necessary for the effects of stress on breast cancer progression, despite its greater affinity to β_2AR compared to norepinephrine. Future studies will characterize the sources of catecholamines that mediate the neural regulation of cancer, including tumor and lymphoid innervation, as well as possible contributions from immune cells.

Conflict of interest

The authors declare no conflicts of interest in relation to this manuscript. The laboratory of Gavin Lambert has recently received research funding from Medtronic. Professor Lambert has acted as a consultant for Medtronic and has received honoraria or travel support for presentations from Pfizer, Wyeth Pharmaceuticals, Servier and Medtronic. Erica Sloan is on the SAB for Cygnal Therapeutics.

Acknowledgments

This work was supported by a National Breast Cancer Foundation Australia postdoctoral fellowship (PF-15-014) and a Monash Interdisciplinary Research Scheme Award to AKW; the David and Lorelle Skewes Foundation, the Peter Mac Foundation, and the National Health and Medical Research Council (APP1147498, APP1098887). GL was supported by a Senior Research Fellowship from the NHMRC (APP1042492). This work was supported by the Victorian Government's Operational Infrastructure Support Program.

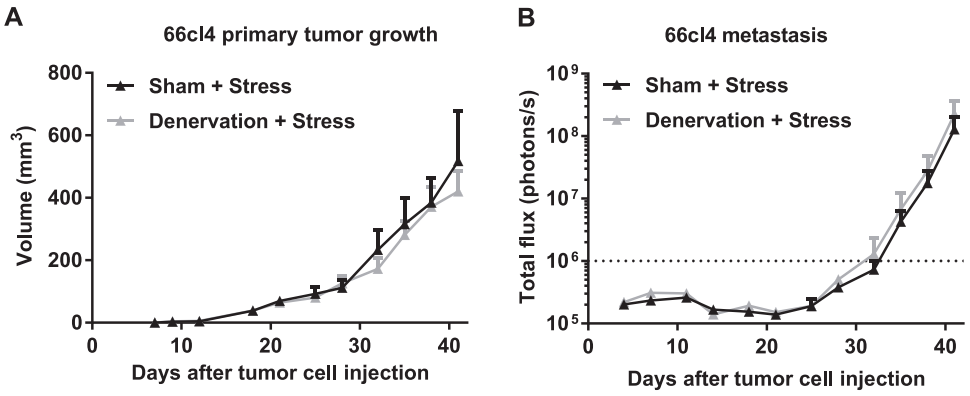

Fig. 4. Cancer growth and metastasis of 66cl4 mammary tumors is not affected by splanchic denervation. Balb/c mice with bilateral splanchnic denervation or sham-operated mice were injected with 66cl4 cells into the 4th left mammary fatpad and exposed to daily restraint stress. A. Primary tumor growth was monitored by caliper. B. Development of distant metastasis was quantified by longitudinal non-invasive bioluminescence imaging.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2018.09.012.

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